

**Focal lesions in toxicity studies;
methods and models**

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ABSTRACT

This project involved the development and evaluation of techniques with regard to their potential use in the identification and investigation of toxic injury, particularly in instances of focal damage, and the development and investigation of a new model for focal renal papillary necrosis (RPN) induced by ethoxyquin. A problem in animal toxicity studies is that compounds such as those which induce RPN may only produce focal lesions, and thus these lesions may be overlooked. Much interest is focused on the development of RPN induced with analgesics and non-steroidal anti-inflammatory drugs (NSAIDS) in both humans and rodents, due to controversy about the pathogenesis of this lesion.

The techniques evaluated for potential use in toxicity studies were: perfusion fixation with and without dye perfusion; histology with evaluation of step-serial sections; urinary sediment analysis; and scanning electron microscopy (SEM) with correlative histology of the same sample. Two well-known models of experimental RPN, 2-bromoethanamine (BEA) and indomethacin, were used to investigate these techniques in the kidney. Urinary sediments and particularly SEM with correlative histology proved useful, and were used in investigating ethoxyquin as a model for RPN. Transmission electron microscopy (TEM) was performed to study the time course of lesion development with ethoxyquin and establish the initial target sites within the papilla. Haematology, clinical chemistry of blood and urine, and histological assessment of all tissues were also conducted to confirm that ethoxyquin did not induce any extra-renal effects which might preclude its use as a model for RPN.

The results suggested that the development of RPN with ethoxyquin follows the same course as the two well-established models, BEA and indomethacin, and the sequence of events reported in man. However, ethoxyquin, in contrast to the two established models but in common with analgesic-induced RPN in man, induces RPN following multiple oral dosing, and has potential as a chronic model for this lesion. Furthermore, the likely mechanism of the development of RPN induced with ethoxyquin is similar to that proposed for analgesics and NSAIDS. The use of ethoxyquin as a model for RPN may therefore help to provide important information for the identification, mechanistic basis and subsequent treatment of RPN in man.

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LIST OF ABBREVIATIONS

AA	= Arachidonic acid
ALP	= Alkaline phosphatase
ALT	= Alanine aminotransferase
AST	= Aspartate aminotransferase
BEA	= 2-bromoethanamine hydrobromide
DMSO	= Dimethyl sulphoxide
DPA	= Diphenylamine
EDTA	= Ethylenediaminetetra-acetic acid
EQ	= Ethoxyquin
EQN	= Ethoxyquin nitroxide
ER	= Endoplasmic reticulum
ERC	= Entorhinal cortex
FAP	= Fatty acid peroxides
GLP	= Good laboratory practice
GSH	= Glutathione (reduced)
H&E	= Haematoxylin and eosin
HETE	= Hydroxyeicosatetraenoic acid
HPETE	= Hydroxyperoxyeicosatetraenoic acid
IM	= Indomethacin
LDH	= Lactate dehydrogenase
NAD	= No abnormalities detected
NSAIDS	= Non-steroidal anti-inflammatory drugs
PG	= Prostaglandin
PGS	= Prostaglandin synthetase
PUFA	= Polyunsaturated fatty acids
RPN	= Renal papillary necrosis
SD	= standard deviation
SEM	= Scanning electron microscopy
SOPs	= Standard operating procedures
TEM	= Transmission electron microscopy

CHAPTER 1. INTRODUCTION

Investigation of the causes, mechanisms of damage and possible treatments of many diseases in man requires animal research and the use of animal models. Research and development in the pharmaceutical industry is a prime example of this; adequate human data are not available for most hazardous substances, so risk analyses must rely on information obtained from laboratory studies of rats or mice. The inference that the results of animal experiments can be applied to humans is a fundamental principle of all toxicological research [Hard et al 1993].

The use of animal models enables the possible mechanisms of diseases such as renal papillary necrosis (RPN) to be investigated and possible treatments or preventive measures to be evaluated [Whittingham et al 1989, Burrell et al 1990]. There is also an important requirement to develop sensitive and specific methods for the early identification of lesions such as RPN, both for animal studies (to help define the spectrum of events and any contributing factors) and for patient care and prognosis [Bach & Bridges 1985, Bach & Hardy 1985, Murray & Brater 1990, Price et al 1996].

This introductory chapter first describes the aim and scope of toxicity studies in the pharmaceutical industry and the role of histopathology within toxicology. The problem of identifying focal lesions in such studies are described and illustrated by discussing two tissues, the brain and the kidney. The chapter then concentrates on the renal papilla and the specific issue of RPN (primarily a problem in man but also reported in domestic animals), the role that experimental models play in studying the mechanisms proposed to be involved in the development of this lesion, and the aims of the project.

1.1 TOXICITY STUDIES

The prime objective of the Pharmaceutical Industry is the production of safe, efficacious drugs. To help achieve this, a great deal of time and effort is spent on pre-clinical toxicity studies before the compound can be used in patients. The aim of such studies is to define the deleterious potential (evaluate the safety) of the compound in a rodent and non-rodent species (usually rat and dog respectively). This is a complex, multi-disciplinary effort involving drug metabolism and pharmacokinetics, clinical examination, clinical chemistry, haematology, biochemistry and pathology.

Studies are conducted by following written protocols in accordance with strict regulatory requirements and guidelines. Drug safety is an important aspect of the research and development of novel pharmaceuticals and it is essential that toxicity studies are conducted to a high scientific and technical standard. There is a legal requirement for safety studies intended to support drug registration to be conducted according to the principles of "Good Laboratory Practice" (GLP). Study protocols are therefore designed to comply with GLP requirements, and detail every aspect of the work to be included in the study, any amendments that were made in this work, and the timescales involved. They are backed up by written Standard Operating Procedures (SOPs) to ensure that all aspects of a study are completed satisfactorily and reliable data are produced. Of equal importance is the need for studies to be designed, conducted and reported to the highest scientific standards in order to accurately define the toxicological profile of a compound and make a reasoned extrapolation of the potential risk to man.

Toxicity studies have 3 principal objectives [Zbinden 1991]. The first of these is to define the spectrum of toxicity of a compound by detection of any adverse effects. The second is to extrapolate the results to predict the likelihood and consequence of such findings in other species, particularly man. The third objective is to predict safe levels of the compound, especially for use in man.

Types of toxicity studies performed can be divided into 2 categories; regulatory studies and mechanistic studies [Klaasen 1986]. Regulatory studies are the standard type of toxicity study and are concerned directly with the toxicity testing of a compound, covering the 3 objectives outlined above, to detect any adverse effects of a drug and the likely risk to man. Regulatory studies can vary in duration from single dose studies to multiple administration studies of up to 2 years duration for rodents, longer for some non-rodent species. Historically, study duration has been divided into four main categories (with some overlap) as detailed below [Klaasen 1986].

- **Acute:** This is defined as single or repeated exposure to a compound over a 24 hour period and is generally the first toxicity test performed on a new compound, usually performed in the rat, mouse and sometimes rabbit. It is designed to provide information on the effects following a single administration of a range of doses, up to and including those causing systemic toxicity. Results obtained from the acute study are important in setting dose levels for subsequent multiple administration studies with the compound.
- **Subacute:** This is designed to obtain information on the toxicity of a compound following repeated administration, usually for up to 14 days, in the same species as

used in acute studies. Subacute studies are aimed at establishing the appropriate dose levels for subsequent subchronic studies.

- **Subchronic:** The duration of subchronic studies can vary from 14 to 90 days, and is of sufficient length to support human administration of the compound. They are usually conducted in two species, rodent and non-rodent (usually rat and dog) by the route of intended exposure in humans.
- **Chronic:** Long-term or chronic studies are performed similarly to subchronic studies except the period of exposure is longer. The length of exposure is partly dependent on the intended period of exposure in humans. If the compound is planned to be used for short periods of time (e.g. antibiotics) a chronic study of six months may be sufficient, performed in a rodent and non-rodent species. However, if the compound has the potential of lifetime exposure in humans a chronic study of up to two years duration (in rodents) will be required. Carcinogenicity studies are a specific type of chronic study. Such studies are designed to assess the carcinogenic potential of a compound and are usually conducted in rats and mice, extending over the average life span of the species (usually 2 years).

Mechanistic toxicity studies are concerned with elucidating the mechanisms by which the drug exerts its toxic effects as there is an increasing requirement by regulatory authorities to do this.

An understanding of the mechanisms of toxic action contributes to the knowledge of basic physiology, pharmacology, cell biology and biochemistry. Results of such studies can lead to the development of sensitive predictive tests which will be useful in obtaining information for risk assessment to support regulatory studies, or rational therapy for toxic symptoms.

A more flexible and scientific approach is necessary to improve the predictive value and cost-effectiveness of toxicological investigations. General features of the investigations in any toxicity study are the large quantity of material to be prepared and examined and the corresponding logistic demands this places on laboratories and scientists. It can be the case that a lot of time and detail is given to some aspects of a study which may not actually add a great deal to the overall findings, so it is important to critically assess techniques or evaluation methods to help decide where best to concentrate efforts. Any developments in either preparatory or examination techniques which help do this are extremely valuable, and constant efforts are made to develop rapid, reliable diagnostic methods.

1.2 ROLE OF HISTOPATHOLOGY

Pathology data make up an essential part of the toxicological information submitted to regulatory authorities to support the registration of a compound [Dua & Jackson 1988]; the interpretation of the pathology and other safety data generated from toxicity studies forms the basis for decisions about the safety of a compound. Histopathology is the final phase of any toxicity study except those solely designed to assess lethality. It involves the detailed examination of the cellular structure of a wide range of tissues for each animal included in a study using gross examination and light microscopy, often also including histochemistry, immunocytochemistry and electron microscopy. At the end of the study period for a particular compound, a complete dissection is carried out, with weighing of major organs and up to 50 tissues being preserved in a suitable fixative, usually 10% neutral buffered formalin. These tissues cover all organ systems. From these, Haematoxylin and Eosin (H&E) stained sections are produced and examined by a pathologist, along with any extra techniques that have been performed (immunocytochemistry, electron microscopy).

Histopathology therefore involves the preparation and examination of a significant quantity of material, particularly in carcinogenicity studies where at least 400 animals are involved. It is important that assessment of tissues for any pathological change is carried out in a reliable, consistent and reproducible manner, and there are various important factors to consider in achieving this.

It is desirable to use a form of grading to categorise any damage seen as this allows dose-responses of any compound-related effects to be studied. The pathologist should have a clear idea of the criteria he or she is going to use for this grading, and an adequate understanding of the pathological process involved. Histological assessment by grading is a subjective procedure open to bias and is therefore often better carried out under "blind" conditions (no prior knowledge of dose groups). However, this in itself can cause problems: some findings reported in a blinded review may be insignificant background changes or normal variations [Black 1991]; or subtle changes may be missed which are only detectable by comparing controls against dose groups [Iatropoulos 1984]. A possible solution to this is to undertake an initial "unblinded" review of a portion of the tissues from control groups to establish background information before starting the blinded assessment [Black 1991].

Another potential source of bias in pathological assessments, which can be a serious problem, is a change in the diagnostic parameters or "diagnostic drift" [Newman 1979, Greaves & Faccini 1984], which can result from factors such as a break in continuity of the assessment or a change in the awareness of the pathologist. It is therefore advisable to re-examine some of the slides to ensure there are no such variations. Peer review of the slides and report by another pathologist is also recommended to minimise any subjective bias and improve the

consistency of the reported findings [Black 1991]. The use of different diagnostic terms can also be a serious source of confusion [Iatropoulos 1984, Dua & Jackson 1988]. To avoid controversy over diagnoses and reduce inconsistencies both within a report and between pathologists, a "Standardised System of Nomenclature and Diagnostic Criteria" has been introduced by the Society of Toxicological Pathologists as a reference guide.

A final, very important, source of bias is sampling [Greaves & Faccini 1984, Eustis et al 1994]. If extra sections have been taken for further examination from selected animals only, for example those with gross lesions or an indication of possible necrosis in the initial section, these animals will have had comparatively more tissue examined than others in the study. To eliminate this bias, either extra sections should be taken from all animals or only one section of tissue examined from those animals which were re-sampled [Eustis et al 1994]. More importantly, this problem can also occur in reverse in that insufficient sections may be examined, which leads to an under-estimate of the presence or extent of any lesions [Dua & Jackson 1988].

This is of particular importance in cases of focal lesions, where the likelihood of identifying the presence of a lesion will vary depending on its size and the frequency with which it occurs. It may be necessary to examine serial sections of the tissue involved to get an accurate picture of the presence and incidence of a particular lesion, for example in the case of renal papillary necrosis with some non-steroidal anti-inflammatory drugs [Arnold et al 1974, Bach & Bridges 1982]. Also, grading of treatment-related changes can also be difficult in cases of focal lesions, since the severity of the effects may not necessarily be related to the size of the lesion.

1.3: FOCAL LESIONS

The problems of accurately identifying the presence and relationship of focal lesions can be illustrated by examining two organs, the brain and kidney. Both are specialised systems involved in maintaining homeostasis and are common target sites for adverse chemical effects.

1.3.1: Brain

The brain is a highly complex organ with anatomical and functional specialisations, one or more of which may be susceptible to chemical injury [Greaves & Faccini 1984, Glaister 1986, Chang 1995]. The brain is selectively protected from polar chemicals by the blood-brain barrier, but is vulnerable to the effects of non-polar chemicals that can cross this barrier [Glaister 1986]. Since pharmaceutical compounds are specifically designed to cross lipid barriers to improve absorption from the gastrointestinal tract, and neurologically active compounds are of great therapeutic interest, it is perhaps not surprising that neurological effects are fairly common in regulatory toxicity studies. Also, the brain is relatively lacking in toxicant metabolising systems compared to other tissues, which can be an advantage or a disadvantage depending on whether the parent compound or a metabolite is the primary toxic molecule [Glaister 1986].

The brain consists of various cell types with different functions and therefore different biochemical targets for toxicants. The neurone is generally considered the most sensitive cell because of the glucose-dependent high metabolic activity associated with active ion transport. Neurones are also particularly susceptible to anoxic effects, either from hypoxic insults (whether from a direct effect on cellular respiration or indirectly by effects on the blood supply) or post-mortem anoxia. Apart from the main cell body, most neurones are associated

with structural entities such as axons, dendrites, neurofilaments and synaptic terminals, all of which are also subject to alterations under toxic conditions [Chang 1995].

The brain poses particular preparation problems because of its susceptibility to post-mortem changes, which can make diagnosis of subtle effects difficult [Duchen 1984, Lamberts & Goldsmith 1986, Greaves & Faccini 1992]. Adequate histopathological assessment of the brain requires appropriate sampling, good fixation and careful histological technique [Greaves & Faccini 1992]. Immersion fixation in formalin is widely used for the brain and is generally regarded as adequate. However, a good knowledge of possible artefacts produced by the process is essential [Duchen 1984], and perfusion fixation may be required for more detailed characterisation of lesions or definition of no-effect levels [Lamberts & Goldsmith 1986, Krinke 1988, Greaves & Faccini 1992]. In animal toxicity studies, particularly when studying agents with activity in the central nervous system, sectioning of the brain may require use of a metal mould or matrix to accurately locate particular cerebral nuclei or fibre tracts at risk of damage.

The use of multiple sections is important for the location of focal lesions in the brain, which may be easily missed due to their location and/or size. Examples include cerebral tumours [Dua & Jackson 1988, Greaves & Faccini 1992], and infarcts (focal areas of ischaemic necrosis) which can occur in the brain of aged rats [Barbolt & Everett 1990] or as a toxic effect [Krinke 1988]. Also, the presence of infarcts in the valves of the heart can cause microthrombi which may detach and lead to infarction in the small vessels of the brain. The correlation of such effects to accurately identify the cause of the brain infarct can therefore be difficult as both may be focal in nature.

As discussed above, the susceptibility of the brain, and particularly neurones, to post-mortem changes can further complicate the identification of focal lesions, since it can be difficult to determine whether an area of neuronal necrosis is due to a toxic effect (ante-mortem necrosis) or to post-mortem autolysis. It is therefore important to fix the tissues as soon as possible after death to help distinguish between the two.

1.3.2: Kidney

The kidney is also a frequent target for the toxic action of chemicals [Bach & Bridges 1982, Glaister 1986]. The main reasons for its susceptibility are related to the large fluid transfers associated with elimination of waste products and xenobiotics and with body water, electrolyte and acid-base homeostasis. The histopathological assessment of the kidney in toxicity studies generally relies on a single section, cut through the middle of the kidney to include the papilla tip [Eustis et al 1994]. The identification of cortical lesions in the kidney is relatively easy since a large area of the cortex is present in a section, but identification of lesions in the papilla is more difficult [Arnold et al 1974, Bach & Bridges 1982, 1985]. Papillary lesions can be missed for several reasons: the lesion may be absent from the standard section examined if this section does not include the papilla tip; if present, there may be insufficient tissue to allow identification of the lesion; or the lesion may be focal, as with focal renal papillary necrosis of the kidney caused by analgesics and non-steroidal anti-inflammatory drugs [Bach & Bridges 1982, 1985], discussed further in Section 1.6.

Histological identification of focal lesions presently relies upon serial or step-serial sections [Arnold et al 1974, Bach & Bridges 1982, 1985, Greaves & Faccini 1992]. However, the preparation and examination of serial sections is a very laborious, time consuming process.

Therefore, any developments which reduce or obviate the need for such a detailed examination and help with the detection of focal lesions would obviously be important [Bach & Bridges 1982 & 1985, Price et al 1996].

The examples discussed above of brain infarcts and renal papillary necrosis are important focal lesions which are induced by several compounds and disease states [Krinke 1988, Ng et al 1989, Parker & Watson 1990]. They are therefore good models to use in the investigation and development of techniques for use in helping to identify focal lesions. Ideally, any developments which are useful should be applicable to any focally damaged tissue and potentially contribute to the knowledge of the lesion in question.

Focal ischaemia (infarction) in the hippocampus region of the brain is a useful model to use for preliminary assessment of techniques (such as perfusion fixation) since it is caused by blockage of the small vessels supplying this area and subsequent ischaemia, and is particularly sensitive to post-mortem changes caused by inadequate preservation techniques. Electron microscopy may be required in toxicity studies for the detection of subtle effects in the brain, but such effects may be masked by post-mortem changes. Development of perfusion techniques using the brain, to optimise tissue preservation, will therefore also be of use for subsequent toxicity studies which require the investigation of nervous tissue. Techniques which appear to be potentially useful using the brain could then be applied and developed using models of renal papillary necrosis, which is an important lesion in the toxicology of analgesics and anti-inflammatory drugs.

1.4 THE RENAL PAPILLA; BLOOD SUPPLY AND SUSCEPTIBILITY

The ways that an individual organ can react to injury are limited and are determined by its anatomy and physiology. A number of diverse agents can therefore produce a similar pattern of injury. The kidney, which receives almost 25% of the cardiac output, is a common target for toxic chemicals since a large amount of any substance present in the blood will be carried to the kidney [Moffat 1982, Bach & Bridges 1985, Bach & Hardy 1985]. The marked susceptibility of this organ to damage is also due to its capacity to extract toxic substances from the filtrate and to accumulate them to critical concentrations in the tubular epithelia [Fent et al 1988]. Active transfer of certain compounds from the plasma to the tubular lumen may lead to higher concentrations in the proximal tubule cell than in the plasma. Also, biotransforming enzymes are present in the kidney which are capable of metabolically activating drugs and chemicals. The main metabolising systems are cytochrome P450 mixed function oxidases in the proximal tubule cells, and prostaglandin endoperoxide synthetase and lipoxygenase in the inner medulla and renal papilla [Glaister 1986, Hawksworth et al 1994]. The metabolising systems in the papilla can co-oxygenate a variety of substances with arachidonic acid to produce reactive metabolites capable of covalently binding to cell macromolecules.

Schematic illustrations of the basic arrangements of the structures and tubules within a kidney lobule and the arrangement of intrarenal blood vessels are shown in Figures 1.1 and 1.2 respectively. The function of the renal papilla is the concentration of urine, therefore any substances or metabolites excreted or generated by the kidney will be concentrated within the tubules of the papilla. Any substance concentrated in the papilla may also modify the distribution of other substances [Burry & Hopkins 1977]. Under normal circumstances, the

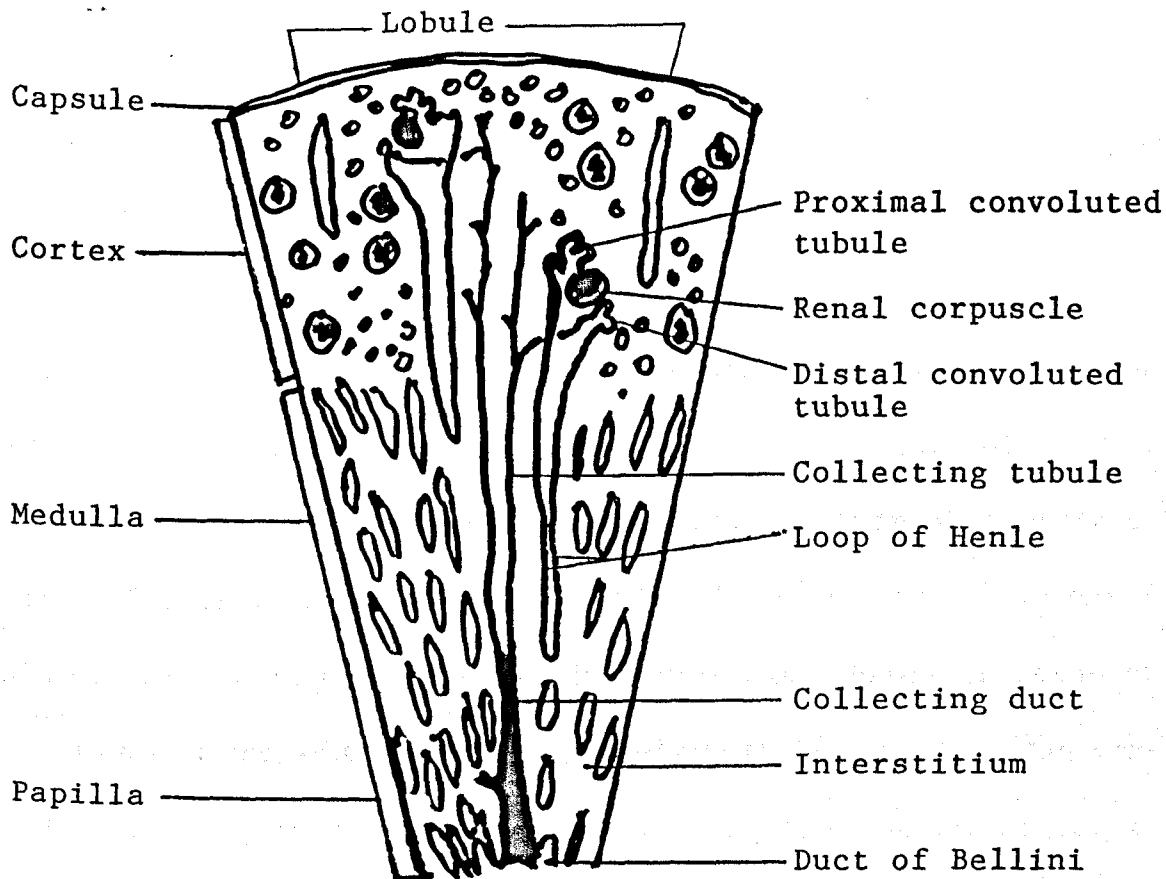


Figure 1.1: Schematic representation of the basic arrangement of nephrons and tubules in a kidney lobule

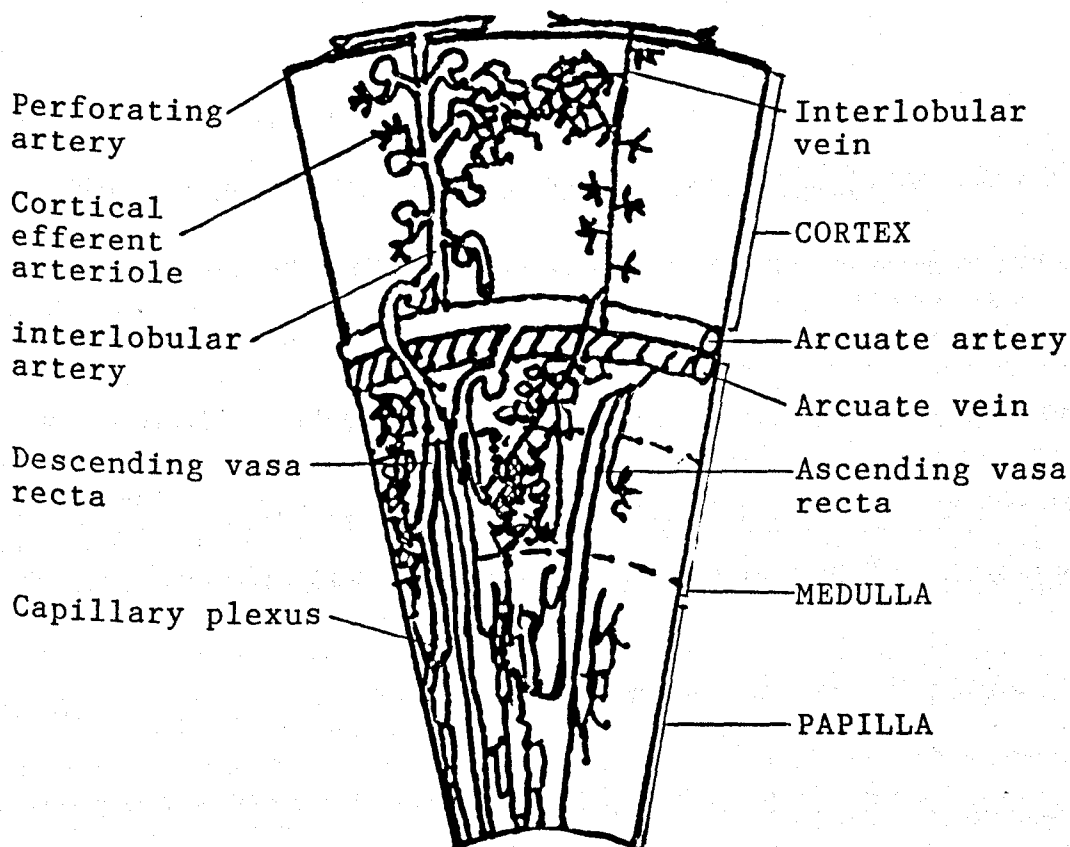


Figure 1.2: Diagram of the arrangement of the intrarenal blood vessels

blood flow to the papilla approximates only 4% of that in the renal cortex due to the unique blood supply of the kidney and its counter-current mechanism.

The blood supply to the papilla is derived from two sources, the vasa recta and the branches of the arteries in the adventitia of the minor calyces [Black 1986]. The vascular bundles formed in the outer medulla decrease in size as they descend towards the papilla tip, where only single vessels remain. The net effect is that the papillary blood supply is poor compared to the rest of the medulla and the kidney cortex. The papilla is therefore considered to be more sensitive to ischaemic damage because of the reduced oxygen delivery to this area. It has also been suggested that the kidney, and particularly the papilla, has a lower rate of protein degradation than the liver and may therefore be more prone to damage from metabolites of compounds such as paracetamol, which covalently bind to tissue proteins [Mudge et al 1978].

1.5 RENAL PAPILLARY NECROSIS IN MAN

Renal papillary necrosis (RPN) in man is a clinical and pathological syndrome which is a common finding in end stage renal disease and occurs as a secondary phenomenon in a number of diseases [Parker & Watson 1990]. It was first described over 100 years ago in a case of ureteric obstruction [Friedreich 1877], and the radiological changes associated with RPN were first described in 1937 [Poynter et al 1974]. However, the characteristic radiological changes (ring shadows, horn-like extensions or clubbing of the calyces, medullary cavities) are not always evident unless RPN has reached the stage where all, or part, of the papilla has become detached [Harrow 1967, Kincaid-Smith 1969]. Recently, renal sonography and computerised tomography have been shown to be useful diagnostic aids which may help in identifying the presence of RPN [Weber 1985, Gupta 1990, Braden 1991, Saifuddin & Bark 1991].

The clinical features of RPN are variable, but usually include fever, flank pain, pyuria, haematuria, low-grade proteinuria and diminished urine concentrating ability. Urinary tract infection is a concomitant finding in the majority of RPN cases. The progression of RPN is insidious and as much as 60-85% of renal function may be compromised before the symptoms become obvious.

The development of RPN in man has been classified into three stages [Burry & Hopkins 1978, Lindvall 1978]:

1. Early papillary necrosis or necrosis in situ, where the damage is less extensive and there is no detachment of the necrotic tissue.
2. Partial or intermediate papillary necrosis, where the central part of the papilla only is necrotic and may detach.
3. Advanced or total papillary necrosis, where the whole papilla is necrotic and often detached from the body of the kidney. The kidney is often reduced in size and the surface may appear ridged.

The earliest morphological features of RPN are degeneration and then necrosis of the fine elements of the medulla (renal medullary interstitial cells, capillaries and thin loops of Henle), followed by progressive necrosis of the covering epithelia of the papilla, collecting ducts and ducts of Bellini. Only the tip of the papilla is affected in the early stages of RPN. This progresses to include the whole papilla with increasing necrosis. Secondary degeneration and glomerular sclerosis occur in the cortex after the entire medulla is damaged. Urothelial hyperplasia is a common sequel to papillary necrosis [Bach & Hardy 1985].

The recognised causes or factors associated with the development of RPN are summarised in Table 1.1. Diabetes mellitus is the most common cause of RPN, being the major factor in 56% of all cases (the incidence of RPN in diabetics is 4 - 5%, compared to an incidence of 0.2 - 0.6% in non-diabetics) [Eknoyan et al 1982, Parker & Watson 1990].

Table 1.1: Underlying causes of renal papillary necrosis

Commonly reported factors	Less common factors
Diabetes mellitus	Chronic alcoholism
Analgesic abuse	Severe jaundice
High dose non-steroidal anti-inflammatory drug treatment	Dehydration in children
Sickle cell haemoglobinopathy	Glomerulonephritis
Dehydrated newborn infants (often jaundiced)	Renal transplantation
Upper urinary tract obstructive uropathy	Prolonged hypotension
Recurrent urinary tract infection	Trauma
Acute pyelonephritis	Renal vein thromboses
Juvenile arthritis	Haemophilia
	Renal artery stenosis
	Pericaliceal haemangioma

[Watanabe & Sakaguchi 1969, Hendry et al 1972, Bannister & Hatcher 1973, Kaude et al 1976, Pandya et al 1976, Lourie et al 1977, Kozlowski et al 1978, Kincaid-Smith 1979 & 1989, Wortmann et al 1980, Bach & Bridges 1982, Chabrel et al 1982, Eknoyan et al 1982, Mitchell et al 1982, Odita et al 1983, Roberts et al 1983, Zielinski 1983, Allen et al 1986, Bailie 1986, Pitts & Van Thiel 1986, Henry et al 1988, Gradus et al 1989, Groop et al 1989, Shapeero 1989, Gupta 1990, Parker & Watson 1990, Lindop et al 1991]

Analgesic abuse accounts for 15% of RPN cases [Eknoyan et al 1982] and is therefore an important cause, but it was not diagnosed until 1953 [Spuhler & Zollinger 1953] and did not emerge as a major aetiological factor until the late 1950's / early 1960's. It has been proposed, and is generally accepted, that the emergence of analgesic abuse as a cause of RPN was a

result of the widespread sale and promotion of analgesic preparations over the preceding 20-30 years [Kincaid-Smith 1979].

1.6 ANALGESIC NEPHROPATHY

Analgesic abuse has been the term traditionally used to describe RPN associated with analgesics, although the more general term “analgesic nephropathy” is often used, which covers the categories of analgesic abuse and high dose non-steroidal anti-inflammatory drug treatment. There is also a well-established association between analgesic abuse, hypertension and gastrointestinal irritation and ulcers [Arnold et al 1974, Wiseman & Reinert 1975, Clive & Stoff 1984, Rainsford 1988, Wrenn et al 1991].

Analgesic nephropathy is one of the few preventable causes of end-stage renal disease, usually associated with the chronic intake of analgesics or, more recently, non-steroidal anti-inflammatory drugs (NSAIDS). It is important that the correct diagnosis is made, since if the intake is discontinued renal function can stabilise or improve [Wilson 1972, Fourie et al 1982], and where renal function is not restored the progression of the disease is generally very slow [Kasanen 1967, Goldberger & Talner 1975]. However, establishing the correct diagnosis can be difficult as patient histories can be unreliable, especially if there is a social stigma associated with abusive or excessive drug consumption.

It is often difficult to establish exactly what drugs have been taken and in what quantity, so the criteria for “abuse” of analgesics is poorly defined. The level of intake necessary to induce RPN has been defined variously as 1-2 tablets daily for more than 2 years [Prescott 1966], 8 or more aspirin or combined analgesic tablets per day [Lalli 1972], a cumulative dose of over 1kg [Kasanen 1967], or between 1 and 35kg [Goldberger & Talner 1975, Gregg et al 1989a].

Reasons underlying an excessive use of analgesics/NSAIDS include such factors as arthritis, headaches or migraines, painful periods, or mood altering effects of constituents such as codeine or caffeine, psycho-social reasons, and family history [Prescott 1966, Murray 1973, Gregg et al 1989a, Dahniya et al 1992].

Generally, there seems to be a greater prevalence of RPN in women than men, and in adults over the age of 40 [Burry 1967, Kincaid-Smith 1979], although there are exceptions [Segasothy et al 1990]. Analgesic nephropathy has been a major problem worldwide [Goldberger & Talner 1975, Prescott 1976, Kincaid-Smith 1989, Elseviers & DeBroe 1994a & b], demonstrating wide geographical variations both between countries (around 1% in the UK, 15% in Australia & 22% in South Africa) and within countries (1% in west & south England, 12% in west Scotland). There are several possible reasons for this difference; variation in the extent of analgesic abuse (the intake of such drugs is reported as higher in the lower social classes), extent of advertising, and climate [Prescott 1966 & 1976, Bach & Bridges 1982, Kincaid-Smith 1989].

There could also be major variations in factors which modify susceptibility to RPN. Elderly patients and those with an underlying renal insufficiency or disorder are considered to be more at risk from analgesic nephropathy as they may already have compromised renal function or consume more analgesics [Black 1986, Murray & Brater 1990, Stilman & Schlesinger 1990].

The incidence of RPN has been generally reported to be higher in countries with hotter climates [Kincaid-Smith et al 1968, Prescott 1966 & 1976, Lalli 1972, Kincaid-Smith 1979 & 1989, Gregg et al 1989a]. It has been proposed that this is because of a contributory dehydration effect on the kidneys, making them more susceptible to the nephrotoxic effects of analgesics and NSAIDS [Cochran et al 1967, Nanra 1976, Prescott 1976]. This hypothesis has

been supported by several research reports implicating dehydration as a contributory factor in RPN seen in premature babies and children [Bannister & Hatcher 1973, Wortmann et al 1980], elderly patients [Stilman & Schlesinger 1990], in horses [Gunson 1983, Gunson & Soma 1983] and domestic cats [Wolf et al 1991a]. Research involving the effects of diuresis and antidiuresis on RPN in rats also supports this association between dehydration and RPN [Fuwa & Waugh 1968, Black 1986].

Whatever the reasons for the differences in distribution of and susceptibility to RPN, and it is likely that multiple factors are involved, it is also likely that the reported incidences of analgesic nephropathy are higher than figures suggest because of the problems in diagnosis and in establishing intake of drugs, as discussed earlier. Analgesic nephropathy continues to be a serious problem in many parts of the world, particularly in Belgium where there has been only a slight decrease in incidence in the last 15 years [Elseviers & DeBroe 1994a & b].

The majority of early reports of RPN implicated phenacetin as the causative agent of papillary necrosis in analgesic mixtures, leading to its withdrawal in the early 1970's [Burry 1967]. The potential nephrotoxicity of the other products was largely overlooked. Phenacetin was replaced by its main metabolite paracetamol and there was an initial drop in the number of reported cases of analgesic-associated RPN, although the incidence did not reduce as much as expected [Nanra 1976, Kincaid-Smith 1989, McCredie & Stewart 1988]. There is a general consensus that the original theory proposing phenacetin as the sole analgesic responsible for RPN was incorrect since RPN continued to be reported with analgesics that did not contain phenacetin. It has also been stated that the risk of RPN with phenacetin is 18-20 times greater than with other analgesics [McCredie et al 1982, McCredie & Stewart 1988], but this quoted risk (and in fact most reported cases of RPN caused by phenacetin) concerned phenacetin as

part of a compound mixture rather than alone. It was therefore unwise to discount the other components of mixed analgesics as factors, particularly since the incidence of analgesic-induced RPN was not markedly reduced with the replacement of phenacetin by paracetamol in compound analgesics.

Combined analgesic preparations have been more commonly associated with RPN, especially those containing aspirin, paracetamol and caffeine. It is proposed that this is due to synergistic, interactive effects of the drugs involved, although combined preparations are also thought to be more habit-forming because of the mood-altering and addictive features of components such as caffeine and codeine [Arnold et al 1977, Duggin 1980, Henry & Tange 1987, Rainsford 1988, Kincaid-Smith 1989, Champion de Crespigny et al 1990]. Powder preparations have been reported to be more potent than tablets because of their more rapid absorption [Prescott 1976, Kincaid-Smith 1989]. However, more cases of RPN may have been reported with powders because they are considered more "acceptable" to take regularly, especially in cases of genuine abuse, and powders are more easily available and therefore purchased more often than tablet preparations [Kincaid-Smith 1989].

It was hoped that the incidence of analgesic-induced RPN would decline following the recognition of this as a cause of end-stage renal failure, increased awareness of the condition and legislation and advice warning against the chronic intake of high doses of analgesics. The prescription and use of NSAIDS increased to replace some uses of analgesics. However, RPN has been increasingly associated with the prescription and intake of high doses of analgesics and NSAIDS for recognised illnesses such as ankylosing spondylitis [Lourie et al 1977] and various types of arthritis in adults [Wiseman & Reinert 1975, Munn et al 1982, Colome-Nafria et al 1991] and rheumatoid arthritis in juveniles [Wortmann et al 1980, Allen et al 1986, Bailie

1986]. Furthermore, cases of RPN have been reported following intake of various NSAIDS at commonly prescribed doses. Those compounds so far associated with RPN include benoxaprofen, fenoprofen, flurbiprofen, naproxen, phenylbutazone, ibuprofen and indomethacin [Arnold 1977, Eknayan et al 1982, Erwin & Jones 1982, Bach & Hardy 1985, Kincaid-Smith 1989, Murray & Brater 1990, Colombe 1991, Colome-Nafria et al 1991, Price et al 1996]. It is therefore clear that analgesic-induced RPN is still an important problem [Mann et al 1993, Elseviers & DeBroe 1994a & b, Price et al 1996]

1.7 RPN IN DOMESTIC ANIMALS

Renal papillary necrosis also occurs in horses, cattle, sheep, goats and dogs [Todd et al 1975, Gunson 1983, Gunson & Soma 1983, Faulkner et al 1984, Wabke 1986], pigs [Gregg 1989b], cats and tigers [Wolf 1991]. Reports of NSAID-induced RPN have been made in various of these "domestic" animals and support the observations in humans. The earliest reports in veterinary literature of a significant correlation between RPN and administration of NSAIDS (phenylbutazone and/or flunixin meglumine) were in horses. A retrospective study of 16 horses where RPN was found at necropsy showed that almost all had been treated with NSAIDS [Gunson 1983]. A second retrospective case control study, where records of drug administration were available, revealed an incidence of 2.6% RPN in all horses necropsied at a large animal clinic [Faulkner et al 1984]; twenty-five of the thirty affected horses had received NSAIDS and the relative risk of horses developing RPN after treatment with these drugs was calculated to be 16.6.

In both studies, a large proportion of the affected horses had been dehydrated or their water intake had been compromised. Furthermore, a subsequent study in horses determined that a reduction in water intake precipitated RPN in 5 horses given normal therapeutic doses of

phenylbutazone [Gunson & Soma 1983]. Similarly, two domestic cats and a tiger pup which developed RPN had been treated with analgesics and/or dehydrated [Wolf 1991a]. These reports add further weight to the hypothesis that dehydration may be an important factor in the development of RPN. All of these reports have also suggested the inhibition of prostaglandin synthesis as a possible key factor (discussed further in 1.9.5).

1.8 EXPERIMENTAL RPN

Renal toxicity is caused by compounds of different chemical structures, acting by a variety of biochemical and/or physiological mechanisms. For assessment of nephrotoxicity, it is necessary to differentiate between functional changes, such as increased water reabsorption following dehydration, and deleterious toxic changes which may or may not be reversible. Toxic agents are characterised by the potential to cause irreversible damage, although they may also have a reversible toxicity. Compounds may first exert their toxicity by means of reversible effects on kidney cell function without cellular destruction or functional impairment [Bach & Bridges 1985]. At higher doses, toxic agents may also lead to pathological changes and cell necrosis with functional compensation for the impairment of the kidney. Alternatively, the damage may be too extensive to allow for functional compensation and renal failure develops. All drug-related nephrotoxicity begins with biochemical and/or physiological alteration of the kidney before leading subsequently to cellular degeneration and necrosis. It is therefore important to detect nephrotoxicity in the early stages of alteration before cell necrosis and consequent functional impairment occurs, so that progression to this stage can be prevented or minimised [Bach & Hardy 1985, Murray & Brater 1990].

Adequate human data are not available for most hazardous substances, so risk analyses must rely on information obtained from laboratory studies of rats or mice. The inference that the

results of animal experiments can be applied to humans is a fundamental principle of all toxicological research [Hard et al 1993]. However, there can be specific cases where rats respond differently to other laboratory species or man, which raise questions regarding the applicability of rat data to other species, including humans [Hard et al 1993]. Although in vivo data are essential to help elucidate the mechanisms of drug-induced RPN, extrapolation of results from animals to man is difficult, and the relevance of using animal models to clarify the factors involved in the development of analgesic associated RPN in humans has been questioned for various reasons as discussed below [Rosner 1976, Molland 1978, Bach & Bridges 1982 & 1985, Bach & Hardy 1985].

The administration of analgesics and NSAIDS to laboratory animals, particularly rats, with the aim of better defining the course of cellular changes has often caused highly variable renal lesions and marked extra-renal toxicity, such as gastrointestinal tract necrosis [Molland 1978, Bach & Bridges 1982 & 1985, Bach & Hardy 1985, Bach 1991]. It has often been difficult to study animal models and interpret research data due to irreproducible or variable lesions, often caused by inadequately identified variables such sex and strain of animal used, route of administration, vehicle, dosage regimen and diet [Bach & Hardy 1985]. Some animal species or strains have been shown to be more resistant to RPN than others [Bokelman et al 1971, Owen & Heywood 1986, Whittingham et al 1989]. However, different strains of rats have often been used in various investigations, with little data produced until recently on inter- and intra-species sensitivity, making it difficult to compare the findings from some publications.

Animal models have been shown to be more susceptible to the gastrointestinal effects of NSAIDS (ulceration, irritation) whilst being relatively resistant to the papillotoxic effects of these compounds [Bach & Bridges 1985, Bach & Hardy 1985, Black 1986]. The major

criticism of the relevance of animal models is that large quantities of analgesics or NSAIDS must generally be administered to rodents to produce the effects seen in man [Molland 1976, 1978, Bilyard et al 1990, Bach & Bridges 1985]. However, it has also been reported that some compounds, given in comparable dosage regimens to those used in man, have produced RPN in rodents [Molland 1976 & 1978, Nanra et al 1980, Burrell et al 1991a]. Furthermore, in those patients who have abused analgesics, their level of intake may well have been in excess of the recommended dose over a period of years.

Laboratory animal models still provide the only means of studying the nephrotoxic effects of these compounds and the early development of RPN. Use of these animal models enables the possible mechanisms of this disease to be investigated [Whittingham et al 1989, Burrell et al 1990]. There is also an important requirement to develop sensitive and specific methods for the early identification of RPN, both for animal studies (to help define the spectrum of events and any contributing factors) and for patient care and prognosis [Bach & Bridges 1985, Bach & Hardy 1985, Murray & Brater 1990, Price et al 1996].

A model which has often been used in studies dealing with RPN, particularly in our laboratories, is the anti-inflammatory agent indomethacin [Arnold et al 1974, Wiseman & Reinert 1975, Jackson & Lawrence 1978, Molland 1978, Munn et al 1982]. This causes acute RPN which is most often focal in its pathological appearance, as is analgesic-induced RPN in man. Indomethacin is therefore a useful model which can assist in studying the mechanisms involved in the development of the lesion, identifying the early stages of damage induced with focal RPN and in assessing and developing techniques which will help in this. However, two problems with using indomethacin are that the RPN induced with this compound is of variable incidence, and it induces marked gastrointestinal effects in rodents after a single dose.

The sensitivity of laboratory animals to the gastrointestinal effects, coupled with their relative resistance to the papillototoxic effects of some NSAIDS and analgesics, has meant that various compounds other than analgesics have also been used to create RPN in rats, without the risk of extrarenal lesions, with the aim of extrapolating the findings to those caused with NSAIDS. 2-Bromoethanamine hydrobromide (BEA) causes extensive RPN reproducibly within 24-48 hours after a single intravenous or intraperitoneal dose, and has allowed the progression of the primary lesion to secondary degenerative changes involving fibrosis, scarring and glomerular sclerosis to be followed. This acute model for RPN shows similarities to changes that have been reported in the clinical condition in human analgesic abusers and in animals dosed chronically with analgesics and NSAIDS [Molland 1978, Elliot 1986, Bach & Bridges 1985, Gregg et al 1989c, 1990a & b, Bach et al 1991]. BEA is therefore commonly used as a model for RPN in studies that attempt to clarify the sequence of events leading to RPN or develop techniques for its early detection.

However, experimental models of RPN, such as indomethacin, are not always reproducible, and the analytical techniques used lack sensitivity so that identifying the events prior to necrosis is very difficult. At present, accurate diagnosis of the presence of focal RPN relies on examination of serial histological sections which is very time consuming [Arnold et al 1974, Bach & Bridges 1982, 1985]. It is therefore important to develop techniques to help improve the detection of this lesion (and focal lesions in other tissues). New models of focal RPN which are more reproducible and lack extra-renal toxicity are also potentially of great value in adding to the information available regarding the development of RPN and the mechanisms involved.

1.9 MECHANISMS OF RPN

Although the drugs which may induce RPN continue to be well documented, there is still much debate about the pathogenesis and possible mechanisms of drug-induced RPN and particularly the early changes which precede development of the lesion. Knowledge of such changes could contribute greatly to the diagnosis and prevention of this cause of renal disease. Several hypotheses have been proposed to explain the development of drug-induced RPN [Duggin 1980, Bach & Bridges 1982, 1984 & 1985, Bach & Hardy 1985, Gregg et al 1989a, Hawksworth et al 1994]; these are discussed below.

1.9.1: Counter-current concentration mechanism

The counter-current concentration mechanism is a widely accepted physiological process. The proposed hypothesis for this as a mechanism for RPN is that, as a result of normal counter-current concentration, a concentration gradient develops for the compound, increasing from the cortex to the papilla, and the concentration achieved in the papilla is then sufficient to exert a direct toxic effect [Ljundquist & Richardson 1966, Burry et al 1977, Bach & Bridges 1982]. This proposed mechanism is supported by the fact that experimental induction of RPN is increased by dehydration [Jackson & Lawrence 1978, Black 1986], and the finding that the initial sites of injury correspond to those areas believed to be sites of maximal concentrating ability, i.e. the papilla tip. This is the case with paracetamol [Molland 1978].

However, there are a number of anomalies such as the distribution of aspirin within the kidney, which is localised within the nephron [Molland 1978]. Also, the validity of this hypothesis is less significant considering the fact that loss of urine concentrating ability is a very early clinical effect in human analgesic abusers and is an early consequence of dosing animals with

analgesics [Bach & Bridges 1984, Henry & Tange 1989]. It has therefore been suggested that active urine concentration may be an important factor, but it does not explain the toxicodynamics or molecular mechanism underlying the lesion [Bach & Hardy 1985].

1.9.2: Ischaemic injury

Medullary ischaemia is often cited as a possible mechanism in the pathogenesis of RPN. As discussed earlier, the medulla, and particularly the papilla, is poorly perfused compared to the cortex and functions at a relatively low oxygen tension, which predisposes it to anoxic injury. Microvascular degeneration and medullary ischaemia have been strongly favoured as the pathophysiological changes that explain the focal nature of RPN [Harrow 1967, Kincaid-Smith et al 1968, Nanra et al 1973, Nanra 1976, Rosner 1976, Bach & Bridges 1985, Heyman et al 1988 & 1991], but microvascular occlusion is not a pre-requisite for acutely induced lesions in animals [Bach et al 1983].

Medullary ischaemia has often been demonstrated in experimental analgesic nephropathy and correlated with evidence of medullary dysfunction, such as impaired concentrating ability and sodium conservation [Nanra et al 1973, Stilman & Schlesinger 1990]. Administration of analgesics and NSAIDS may alter renal haemodynamics by virtue of their inhibitory effect on vasodilatory prostaglandins, described further in 1.9.5 [Arnold et al 1974, Jackson & Lawrence 1978, Fong & Cohen 1982, Bach & Bridges 1985, Stilman & Schlesinger 1990, Wrenn et al 1991]. Also, it has been suggested that the gastrointestinal ulceration induced by NSAIDS and analgesics may predispose the papilla to ischaemia because of lowered tissue perfusion and dehydration [Wrenn et al 1991]. It has been proposed that any alteration in renal haemodynamics resulting in decreased papillary blood flow would lead to ischaemia [Molland 1978, Fong & Cohen 1982]. Cristol et al [1993] demonstrated that the formation of the

endogenous vasodilator nitric oxide was also important, not only for the maintenance of renal blood flow but also for the recovery and maintenance of renal blood flow after ischaemic injury. However, there is conflicting evidence regarding the effects of papillotoxins on blood flow; no net change in blood flow was observed with the experimental model, BEA [Solez et al 1974, Sabatini 1984].

Medullary ischaemia is therefore often cited as a possible mechanism in the pathogenesis of RPN, but it is very difficult to separate the microvascular changes from the other degenerative pathology. It is therefore impossible to determine whether the microvascular changes seen in chronically developing RPN in man and experimental animals are the primary pathological event, preceding RPN, if they run parallel with, or if they are secondary to necrosis.

1.9.3: Immunotoxic effects

Early reports suggested an immunological basis for the pathogenesis of RPN because of the slow onset of the lesion and the large variability of human analgesic abusers and experimental animals exposed to analgesics and NSAIDS [Harrow 1967, Bach & Bridges 1984]. However, there is no convincing evidence to support this view and it is now generally accepted that this is not involved in the pathogenesis of RPN [Bach & Bridges 1984 & 1985, Bach & Hardy 1985].

1.9.4: Changes in intermediary metabolism

High concentrations of several analgesics have been reported to depress or disrupt renal intermediary metabolism [Mudge et al 1978, Bach & Bridges 1985]. However, since the investigations reported were confined to mitochondria of cortical or cortical/medullary origin, and the biochemical functions of cortical and medullary tissue are very different, it has been

proposed that they are not necessarily relevant to RPN, which is a medullary lesion. In order to clarify whether changes in intermediary metabolism are involved in the development of RPN, it would be necessary to look at the effects of compounds on the medullary cell types affected in the early stages of this lesion [Bach & Bridges 1985]. However, it may be that the reported effects on intermediary metabolism are involved in rendering the kidney more susceptible to the papillotoxic effects of the compounds.

1.9.5: Inhibition of prostaglandin synthesis

Another proposed mechanism of RPN is a direct pharmacological alteration of renal medullary (papillary) prostaglandin synthesis. Prostaglandins are ubiquitous substances which function mostly as "local hormones", or prostanoids [Molland 1978, Clive & Stoff 1984, Stilman & Schlesinger 1990]. They are unsaturated fatty acid compounds derived from essential fatty acids; the most important precursor of prostaglandins is arachidonic acid. Their biological activity is exerted primarily at the site of synthesis, since they have a short half-life in the circulation [Janszen & Nugteren 1972, Muirhead & Pitcock 1980]. Furthermore, they are synthesised immediately before release (on demand), initiated by the liberation of arachidonic acid from the phospholipid pool of cell membranes. Figure 1.3 is a schematic representation of the arachidonic acid cascade and formation of prostaglandins, including the proposed pathways for the oxidative activation of NSAIDS and analgesics (discussed below).

The kidney is extremely active in the synthesis and metabolism of prostaglandins. Prostaglandin synthesis occurs in both the cortex and medulla; the major medullary prostaglandin is PGE₂, produced in the renal medullary interstitial cells [Muirhead et al 1972a, Zusman 1977]. This prostaglandin directly contributes to the effects of natriuresis by inhibiting reabsorption of sodium and chlorine [Muirhead et al 1972b, Muirhead & Pitcock 1980], but its

major role is as a vasodilator, helping to maintain adequate blood flow. Prostaglandins have only a minor role under normal physiological conditions but are crucial for the maintenance of renal perfusion following a haemodynamic insult [Henrich et al 1978, Cristol et al 1993].

Analgesics and NSAIDS produce much of their therapeutic and adverse effects through inhibition of prostaglandin synthesis, by inhibiting cyclo-oxygenase (Figure 1.3); indomethacin and aspirin are both well-documented inhibitors of prostaglandin synthesis [Molland 1978, Dunn & Zambraski 1980, Clive & Stoff 1984, Marnett et al 1984, Zenser & Davies 1984, Bach & Bridges 1985, Gregg et al 1989c, Hawksworth et al 1994]. It has therefore been proposed that it is this inhibition of prostaglandin synthesis which is the major factor in the development of RPN. Inhibition may reduce vasodilation, as discussed earlier, and may lead to the metabolism of arachidonic acid via the lipoxygenase pathway (Figure 1.3, discussed further below), resulting in the production of leukotrienes which can have profound effects on renal haemodynamics. However, the perturbation of the renal system may be very complex, and factors other than the inhibition of PGE_2 may be involved in the development of this lesion [Dunn & Zambraski 1980, Bach & Bridges 1984 & 1985, Bach & Hardy 1985, Kirkova et al 1995].

1.9.6: Metabolic activation

Metabolic activation of chemicals to reactive intermediates which injure cells is recognised to be a primary event mediating the toxicity of a variety of industrial, environmental and therapeutic chemicals [Bach & Hardy 1985]. Metabolic activation of xenobiotics is thought to occur via the formation of free radicals, strongly electrophilic intermediates or chemically unstable metabolites which give rise to reactive and possibly damaging products (reactive intermediates). Endogenous material such as polyunsaturated fatty acids, of which there are

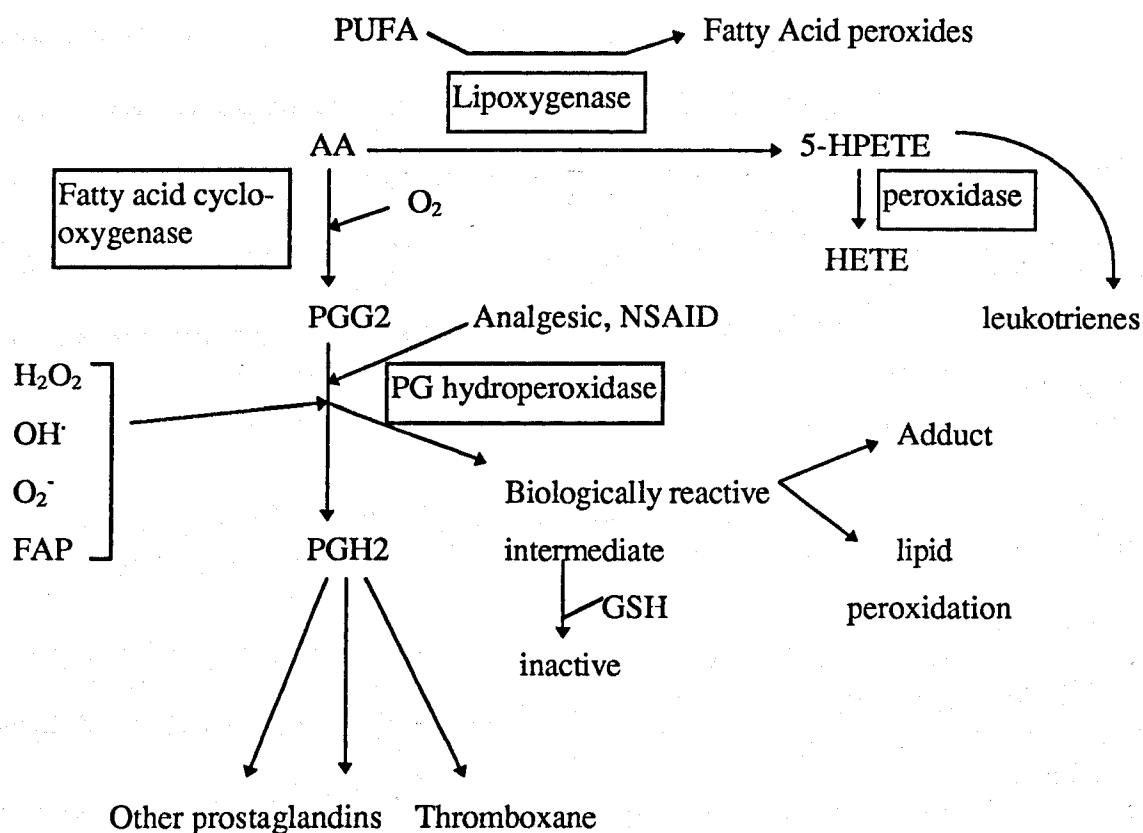
high levels in the papilla, can also be converted to potentially damaging reactive intermediates under certain circumstances [Bach & Bridges 1985].

As mentioned above, the reactive intermediates generated by metabolic activation are usually strongly electrophilic. Proteins, lipids and nucleic acids contain nucleophilic regions and are therefore targets for these electrophilic intermediates, and the reactive metabolites covalently bond to cellular macromolecules. Marked depletion of renal glutathione (GSH) and extensive covalent binding in the papilla and cortex have been reported after a single high dose of paracetamol [Mudge et al 1978, Mohandas et al 1984]. Also, paracetamol has been shown to bind covalently to proteins only in those tissues which exhibited cellular damage (kidney, liver and lung) [Bartolone et al 1989].

Metabolic activation of xenobiotics can occur in the kidney via cytochrome P450, glutathione transferase, prostaglandin synthetase or lipoxygenase. However, the medulla has no detectable P450 activity [Zenser & Davies 1984], so it is unlikely that this enzyme is involved. Levels of reduced glutathione (GSH) are lowest in the inner medulla, so the activity of the glutathione-related enzymes in the inner medulla and papilla would be expected to cause little interference to prostaglandin synthetase-mediated co-oxygenation and metabolic activation [Mohandas et al 1984]. Metabolic activation could therefore occur via the prostaglandin synthetase (PGS) or lipoxygenase pathways. These involve the co-oxygenation of xenobiotics in association with the metabolism of arachidonic acid (Figure 1.3).

Much work has concentrated on the role of PGS in the development of RPN [Zenser et al 1983, Marnett et al 1984, Mohandas et al 1984, Bach & Bridges 1984 & 1985, Bach & Hardy 1985, Gregg et al 1989a, Bach 1991, Hawksworth et al 1994, Stewart et al 1994]. PGS is

Figure 1.3: Schematic representation of the arachidonic acid cascade including the proposed pathways for the oxidative activation of NSAIDs and analgesics, and the generation of free radicals via lipid peroxidation



Abbreviations:

AA = arachidonic acid; GSH = glutathione; HETE = hydroxyeicosatetraenoic acid;

HPETE = hydroxyperoxyeicosatetraenoic acid;

NSAID = non-steroidal anti-inflammatory drug; $O_2^{\cdot -}$ = superoxide anion;

OH^{\cdot} = hydroxyl radical; PG = prostaglandin; PUFA = polyunsaturated fatty acid.

FAP = fatty acid peroxides; PGG2, PGH2 = prostaglandins G2 and H2

present in significant quantities in the papilla and medulla, and displays an increasing gradient from the cortex to the medulla. Recently, increasing attention has been given to the role of lipoxygenases in RPN, which have also been shown to display a marked cortex to medullary gradient [Bach & Bridges 1985, Miyazawa et al 1985, Kirkova et al 1992 & 1995, Hawksworth et al 1994, Stewart et al 1994]. Furthermore, it has been reported that the arachidonic acid-dependent co-oxygenation of compounds in the human kidney occurs mainly via lipoxygenases, whereas in rat this occurs via PGS and lipoxygenase, suggesting a species difference in the renal metabolism of these compounds [Hawksworth et al 1994, Stewart et al 1994].

The presence of high levels of polyunsaturated fatty acids in the papilla, mainly in the interstitial cells, predisposes these cells to lipid peroxidation in the presence of locally generated reactive intermediates. The reactive intermediates produced via PGS or lipoxygenase therefore have the potential to cause lipid peroxidation and lead to cell death [Bach & Bridges 1984]. Normally, reduced glutathione (GSH) protects the cell from alkylating agents and lipid peroxidation, as shown in Figure 1.3, but the medulla and papilla have low levels of endogenous GSH. It has been suggested that the low levels of reduced glutathione may render the inner medullary region particularly vulnerable to damage from metabolic activation, since once reactive intermediates are generated these will not be readily inactivated [Duggin 1980, Mohandas et al 1984, Gregg et al 1989a].

Metabolic activation is therefore currently regarded as the most likely mechanism for analgesic-induced RPN, but it is not certain whether this occurs by the PGS or lipoxygenase pathways, or a combination of effects. It is also possible that a number of these proposed mechanisms (medullary ischaemia, intermediary metabolism, inhibition of prostaglandins and

metabolic activation), acting in combination or as a cascade effect, are responsible for the development of RPN.

There is therefore still great interest and research concerning the possible mechanisms leading to the development of renal papillary necrosis which remains an important cause of end-stage renal failure in man. There is also a need to develop techniques which aid in the early diagnosis of RPN. Furthermore, there is an obvious need to identify anti-inflammatory and analgesic compounds with the lowest possible risk of producing RPN. It is clear that there is a fine balance between the benefits and harmful effects of these compounds; an understanding of the mechanisms involved in the latter, and advances in the early diagnosis of any lesions, will help in both patient care and in developing drugs with a lower risk of RPN.

1.10 AIMS OF THIS STUDY

The aims of the first part of this study were to develop and evaluate techniques which may help in the diagnosis of toxic injury, particularly in instances of focal damage, and assess the applicability of these to toxicity studies. The brain, specifically the hippocampus, was used as the preliminary model for assessing initial techniques since it is a well-known site of focal ischaemia [Coyle 1976 & 1978, Krinke 1988, Ng et al 1989] and is particularly susceptible to artefactual damage caused by inadequate preservation techniques. The brain studies were conducted as part of the M.Phil phase of these studies; only work which led into techniques used in the kidney has been included.

Those techniques which showed promise with the brain and were applicable to other tissues were then evaluated, along with further techniques, for use in the kidney, using 2 well-known models of experimental RPN, BEA and indomethacin. RPN is an important lesion in the

toxicology of analgesics and one in which the identification of focal lesions has caused problems. Once useful techniques were identified, these were applied to help investigate a new model of experimental renal papillary necrosis. The results obtained from this could then be compared with those obtained with the two well-established models.

The second part of this study was aimed at developing a new model of RPN, using the antioxidant ethoxyquin. The aims of developing this new model were two-fold: i) to develop a reliable, reproducible experimental model for focal RPN, with no/minimal extra-renal toxicity, and ii) to compare the progression and features of the RPN induced with ethoxyquin with that seen with the two other experimental models (BEA and indomethacin) and in man, thereby contributing to knowledge of the pathogenesis and possible mechanisms of this type of damage.

CHAPTER 2. PRELIMINARY BRAIN WORK

2.1 INTRODUCTION

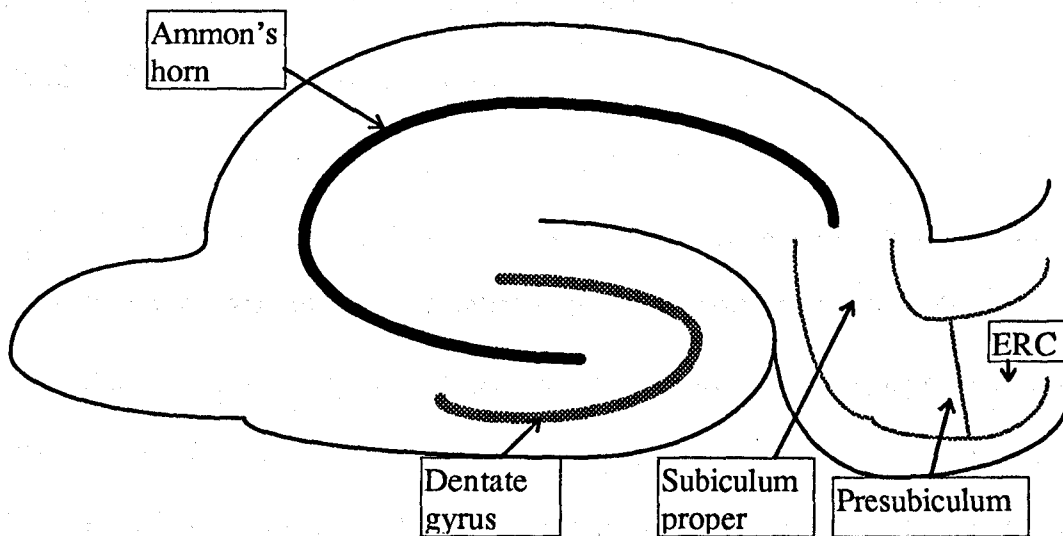
As discussed in the introductory chapter of the thesis, histological identification of focal lesions presently relies upon serial or step-serial sections of the tissue in question, which is a very laborious process. Developments in techniques which help reduce this need would be important assets in both histopathology and toxicity studies. Furthermore, optimal preservation of tissues is important for histopathological assessment, particularly electron microscopy, where post-mortem changes may mask an underlying treatment-related effect or make diagnosis difficult.

The hippocampus region of the brain is a useful model to use for preliminary assessment of techniques aimed at optimising tissue preservation and visualising the vasculature of a tissue, as it is a clearly structured part of the brain which is sensitive to post-mortem changes caused by inadequate preservation techniques and is a potential site for focal ischaemia [Walaas 1983, Krinke 1988]. Also, since the brain is an important target organ in toxicity studies, development of perfusion techniques using the brain will also be of use for subsequent toxicity studies where investigation of nervous tissue is required. The work described in this chapter was conducted as part of the M.Phil studies; only the most relevant work, leading onto work conducted in the kidney, is included.

The hippocampus is a simplified, phylogenetically old part of the cerebral cortex with a unique structural organisation, which is a common site for anoxic damage in the brain [Walaas 1983, Krinke 1988]. The characteristics of hippocampal nervous systems have been investigated in the mechanism of action of various toxicants [Walaas 1983]. The hippocampal region occupies most of the ventroposterior and ventrolateral walls of the cerebral cortex. Six distinct structures make up

the hippocampal region (Figure 2.1): the entorhinal cortex (ERC); parasubiculum; presubiculum; subiculum proper; Ammon's Horn (CA1-CA3); dentate gyrus. The last two structures comprise the hippocampus proper - most studies have been concerned with these areas. Walaas [1983] has written a comprehensive description of the hippocampus and its anatomical structure.

Figure 2.1: Structure of the hippocampus region



Adequate fixation of tissues is vitally important for obtaining good morphological preservation of structures at both light and electron microscope levels. The brain is particularly susceptible to post-mortem necrosis, so rapid fixation is vital. Lamberts & Goldsmith [1986] compared the effects of various fixatives and methods on the ultrastructural morphology of rat brain. They preserved brains by vascular perfusion and/or immersion in nine different fixatives. They found that immersion fixation was best in the median eminence (outside the blood-brain barrier) and close to the hypothalamus surface. Perfusion fixation gave adequate preservation of the arcuate nucleus and tissue within the blood-brain barrier. Perfusion fixation is used in the majority of studies concerning the central nervous system and particularly the hippocampus, although this can cause problems in toxicological studies if any other organs are required to be sampled unfixed (e.g. for biochemistry).

Extensive light and electron microscope investigations have been carried out to obtain information regarding cellular structure and interconnections within the hippocampus in various species, with the aim of extrapolating the findings to human hippocampal structure and establishing the relevance of using the various animal models. Frotscher et al [1988] compared the fine structure of identified neurons in the hippocampus of the baboon to that in commonly studied small laboratory animals. They found, perhaps as expected, that a much more complex dendritic pattern and synaptic organisation of these neurons exists in primates.

Despite this observation, the hippocampal formation of small laboratory animals is still used as a model structure for studies of general neurobiological phenomena due to the relative simplicity of neuronal composition, with the main cell types arranged in densely packed layers, and the fact that well known afferents terminate in the hippocampus and dentate gyrus in a laminated, almost non-overlapping manner. Ng et al [1989] looked at the frequency and distribution of changes in the hippocampus from hypoxic insults, comparing man with rat. They compared cardiac arrest, hypoglycaemia and status epilepticus as the cause of insult. They found the CA1 region was the most frequently affected in hypoglycaemia in man. In rats, CA1 and CA4 were damaged first after global ischaemia and status epilepticus, with dentate gyrus affected first in hypoglycaemia. The pattern of selective damage was therefore similar in man and rat, supporting the view that rats are a useful model for hippocampal damage.

To enable damage observed in hippocampal cells, particularly with anoxia, to be correlated with changes in blood supply, knowledge of the vascularisation of the hippocampus is necessary. Coyle [1976 & 1978] highlighted the vascular patterns of the rat hippocampal formation and the spatial features, mapping the distribution of the major arteries and veins using silicone injections and

double injections of indian ink and fluorescent material. The aim of Coyle's studies was to determine which arteries and veins could be paired and to map spatial positions to gain better impressions of vascular and neuronal relationships.

Coyle [1976 & 1978] found the CA1 region of the hippocampus to be the most poorly vascularised and the outer 2/3 of the dentate gyrus to be more vascular than the inner portion. He proposed that it is this poor vascularity that predisposes the CA1 area of the hippocampus to injury under certain circumstances, eg. anoxia. Krinke [1988] emphasised that the hippocampus is very vulnerable to secondary ischaemic effects (after damage has occurred in other areas), and interpretation of hippocampal lesions is therefore frequently controversial. As a general rule, CA1-3 is most susceptible in secondary anoxic ischaemia, while CA4 and the dentate gyrus are most affected by primary toxic effects of certain compounds.

The hippocampus is therefore a highly sensitive area to the effects of anoxia induced by various disease states and toxic substances, but the precise mechanisms of action on the hippocampus are largely unknown. The use of animal models for the type of hippocampal damage seen in humans attempts to; (i) clarify the causes of cell damage, and (ii) provide suggestions for treatment or prevention of damage in susceptible individuals. Research on the functioning and morphological appearance of the various structures within the hippocampus in normal and diseased animals continues to be of value in understanding and elucidating the mechanisms of damage.

The aim of the work detailed in this chapter was to evaluate various techniques which may assist in studying focal lesions, using the brain as a preliminary model. The hippocampus is a potential site for focal ischaemic lesions in the brain and is very sensitive to the effects of poor or sub-optimal fixation, due to its susceptibility to post-mortem changes via anoxia, as discussed earlier. Perfusion

fixation provides a method for optimal preservation, but the quality of the results obtained is dependent on the flow rate used. The effects of perfusion fixation at various flow rate were therefore assessed to determine the optimum flow rate for preservation of the hippocampal cells. Dyes have often been used to study the vasculature of tissues and vascular damage [Coyle 1976 & 1978]. Perfusion with dyes to visualise the vasculature of the hippocampus was therefore evaluated to assess its potential use in investigating the involvement of the vasculature in the development of lesions in future studies with the brain or in other tissues where this might be an issue. The techniques which proved useful with the brain were then applied and developed for the kidney, another site of focal necrosis which causes problems in pathological evaluation.

2.2 MATERIALS & METHODS

2.2.1: Materials

2.2.1.1: Chemicals and Suppliers

All buffer salts, formaldehyde (40% stock), alcohol, xylene, cedarwood oil and paraffin wax were obtained from BDH Merck Ltd., Hunter Boulevard, Magna Park, Lutterworth, Leicestershire. Neutral buffered formalin (10%) and all histological dyes used in the Haematoxylin & Eosin staining and dye perfusion methods were obtained from Pioneer Research Chemicals, Unit 7, Commerce Park, Whitehall Industrial Estate, Colchester, Essex.

Electron microscopy fixatives, chemicals, and grids were obtained from Agar Scientific Ltd, Stansted Rd., Essex, U.K. Dyes used in the toluidine blue staining method were obtained from BDH Merck. "Sagatal" anaesthetic was obtained from Rhone- Merieux, Spire Green Lane, Harlow, Essex. Heparin ("Monoparin") and local anaesthetic ("Lignavet"), used in perfusion experiments, were obtained from CP Pharmaceuticals Ltd. Wrexham, and C-Vet Ltd. Bury St. Edmunds, respectively.

2.2.1.2: Animals, housing conditions and dosing regimes

All rats used in the experiments in this chapter were obtained from Charles River (UK) Ltd., Manston Rd., Margate, Kent. Animals were housed at a temperature of $21 \pm 2^{\circ}\text{C}$, relative humidity of $55 \pm 10\%$ and 12 hours light/12 hours dark lighting. Animals were fed with a standard certified rodent diet (RMM diet, Special Diet Services, Cambs., UK), both this and water were provided *ad libitum*.

2.2.2: Perfusion fixation

Each rat (weighing approximately 400g) was terminally anaesthetised with sodium pentobarbitone (Sagatal, 0.2mL/kg) and the body cavity opened to expose the beating heart. Using a syringe, 1mL of prewash solution 1 (0.9% saline containing heparin (25 units/mL), local anaesthetic ("Lignavet", 0.4mg/mL) and sodium nitrite (10mg/mL)) was injected into the left ventricle, simultaneously cutting the right atrium. The abdominal aorta was tightly clamped above the diaphragm using artery forceps to allow the flow of perfusion solutions through the upper body only. A 25 gauge cannula was inserted into the ventricle in the origin of the aorta, clamped in place and 50mL of prewash solution 2 (0.9% saline containing heparin (25 units/mL) and Lignavet (0.4mg/mL)) passed through the heart, flushing out any remaining blood, using a Wellmed syringe pump set at the prescribed flow rate; five different flow rates were used for comparison - 22, 11, 9, 7.5 and 5 mL/minute. One hundred mL of Karnovsky's fixative (0.1M phosphate buffer (pH 7.4) containing 2% formaldehyde and 2.5% glutaraldehyde) was then passed through the heart via the same cannula and flow rate.

After successful perfusion, the brain was removed and the hippocampus region was subdivided for standard histological and electron microscopical (EM) examination to evaluate the effect of the perfusion rate used on the morphological structure. To study the effect of immersion fixation, the hippocampus from one animal was removed without being perfused, subdivided for histology and EM examination, and processed in the same way as the perfused samples.

Tissue for histology was immersed in Karnovsky's fixative overnight and processed to paraffin wax using a Bayer VIP automated tissue processor (50, 80, 95 and 2 x 100% ethanol, 3 x xylene, 1 hour each at 40°C; paraffin wax 3 x 2 hours at 60°C). Tissues were then embedded into paraffin blocks using a Tissue-Tek embedding centre. 7µm sections were cut and stained with Haematoxylin and Eosin (H&E, method in Appendix 1) for evaluation. Tissue for EM was

subdivided and immersed in Karnovsky's fixative for a further 2 hours. Tissue pieces were then post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer pH 7.4 for 1 hour and rinsed in 0.1M phosphate buffer. Samples were then dehydrated through graded alcohols to 1,2 epoxypropane (25, 50, 75 and 100% alcohol, 15 min each, 30 min 1,2 epoxypropane), and infiltrated in CY212 resin overnight before embedding and polymerisation in araldite blocks (48h at 60°C).

Semi-thin (1µm) and thin (60-90nm) sections were cut transversely through the hippocampus using a Reichert Ultracut E ultramicrotome. Semi-thin sections were placed on glass slides, stained with 1% toluidine blue in 1% borax (method in Appendix 1) and the structure observed with a light microscope. Thin sections were collected on 3mm copper mesh grids, stained with uranyl acetate and lead citrate and observed using a Siemens 102 transmission electron microscope at an accelerating voltage of 80kV.

2.2.3: Vascular visualisation

This was a modification of the brain perfusion method detailed in 2.2.2; the same solutions were used for perfusion. Animals were perfused as in the method outlined in 2.2.2 with both prewash solutions, then perfused with either 50mL of fixative solution containing dye, or 50mL of dye alone. The dyes used for perfusion to assess their applicability for visualisation of the vascular supply were indian ink, carmine, neutral fast red, methylene blue and permanent ink, used separately. The dyes were chosen either on the basis of previous reported use of these dyes or the size of dye particle (dyes composed of small dye particles were preferred, to ensure they could be perfused into small capillaries).

After perfusion was complete, the brains were removed and immersed in Karnovsky's solution for 4 hours to complete the fixation process. Whole brains or 1mm-thick sections through the

hippocampal region (cut using a "Harvard" rat brain matrix and skin graft blades) were dehydrated by hand through graded alcohols (25, 50, 75, 90 and 100% 30 mins each) and cleared in several changes of cedarwood oil for at least 48 hours. Slices were placed on glass slides, immersed in cedarwood oil and the extent of vasculature successfully perfused was observed with a dissecting microscope. In order to correlate this information with histology, the most successfully perfused of these 1mm slices were embedded in paraffin wax using a Tissue Tek embedding centre and 4µm sections cut and stained with Haematoxylin and Eosin for observation. Some whole brains perfused with dye were left intact, dehydrated by hand as above and cleared in cedarwood oil to gain an overall picture of brain vasculature. The remaining whole brains were sliced and processed to paraffin block as described above.

2.2.4: Retrospective electron microscopy

Rat brain tissue previously fixed and stored in 10% neutral buffered formalin for 3 months was used to assess the quality of preservation for potential retrospective examination of structurally altered or necrotic tissue. The brain was subdivided, using a "Harvard" rat brain matrix and skin graft blades, into 1mm thick slices and pieces of hippocampus dissected out. Tissue pieces were then dehydrated and embedded in araldite resin blocks for electron microscopy as described in 2.2.2. Thin (60nm) sections for electron microscopy were cut using a Reichert ultramicrotome, mounted on copper mesh grids and observed using a Siemens 102 electron microscope at 80kV.

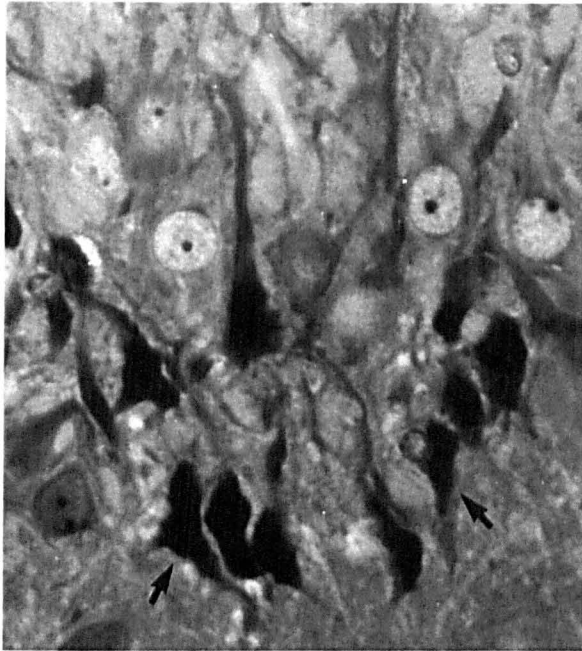


Figure 2.2.: Toluidine blue 1μm resin section (x600) of immersion-fixed pyramidal cells (dark cells arrowed).



Figure 2.3: H&E stained paraffin section of hippocampus, perfused at 22mL/min (x45). Note large spaces (arrowed).

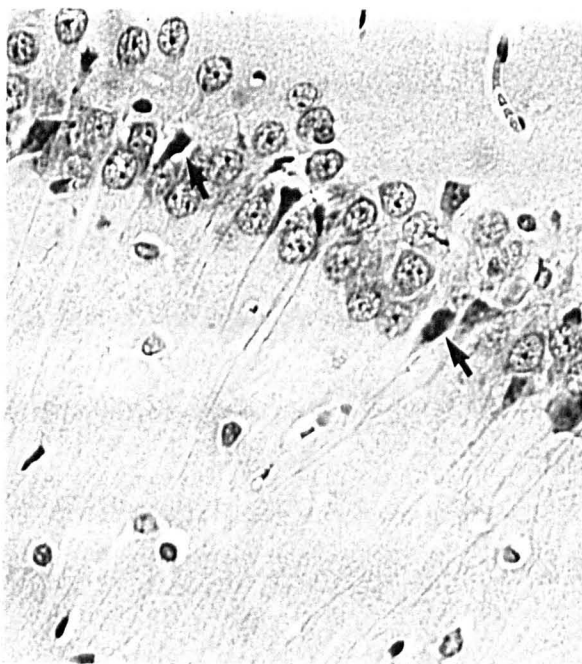


Figure 2.4: H&E stained section of pyramidal cells, perfused at 22mL/min. Note shrunken cells (arrowed). (x300)



Figure 2.5: Electron micrograph of endothelial cell from 9mL/min perfused brain (x25,000).

2.3 RESULTS

2.3.1: Immersion fixation

Light microscopy showed evidence of widespread shrinkage of pyramidal cells in the CA system and dentate gyrus. These irregular, densely stained cells were identified as containing pyknotic nuclei (Figure 2.2). Endothelial cells and vessel contents appeared shrunken. Electron microscopy confirmed poor ultrastructural preservation of pyramidal cells within the hippocampus. This was indicated by cells at various stages of autolysis, namely swollen mitochondria and cisternae of the endoplasmic reticulum (ER) and condensation of chromatin in nuclei. Electron microscopy also confirmed the shrinkage of endothelial cells seen at the light level.

2.3.2: Perfusion fixation

At a perfusion rate of 22mL/min, light microscopy showed considerable perfusion artefacts, specifically dilation of the lateral ventricle and the vessels in the molecular layer of the hippocampus, detachment of the corpus callosum and shrinkage of cells in the CA system of the hippocampus and the dentate gyrus (Table 2.1, Figs 2.3, 2.4). Similar artefacts were seen at a perfusion rate of 11mL/min, although not as pronounced.

A perfusion rate of 9mL/min showed reduced perfusion artefact with only slight detachment of the corpus callosum and some enlargement of vessels by light microscopy. Electron microscopy showed good preservation of endothelial cells, cell membranes and mitochondria with little shrinkage (Table 2.1, Fig. 2.5) At a perfusion rate of 7.5mL/min, light microscopy showed no dilation of ventricles or blood vessels and very good preservation of pyramidal cells with minimal shrinkage (Fig. 2.6). Electron microscopy confirmed excellent preservation of pyramidal cells and contents (Fig. 2.7). Glial cells and endothelial cells were also very well preserved (Fig. 2.8).

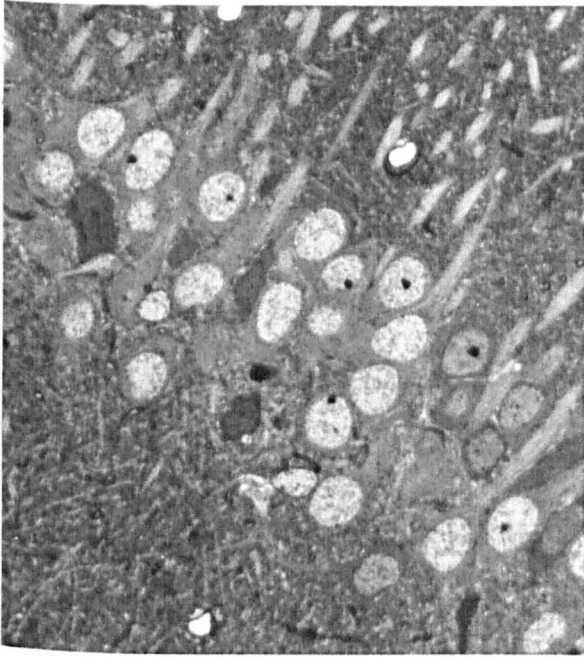


Figure 2.6 Toluidine blue-stained 1 μ m resin section from 7.5ml/min hippocampus (x500).

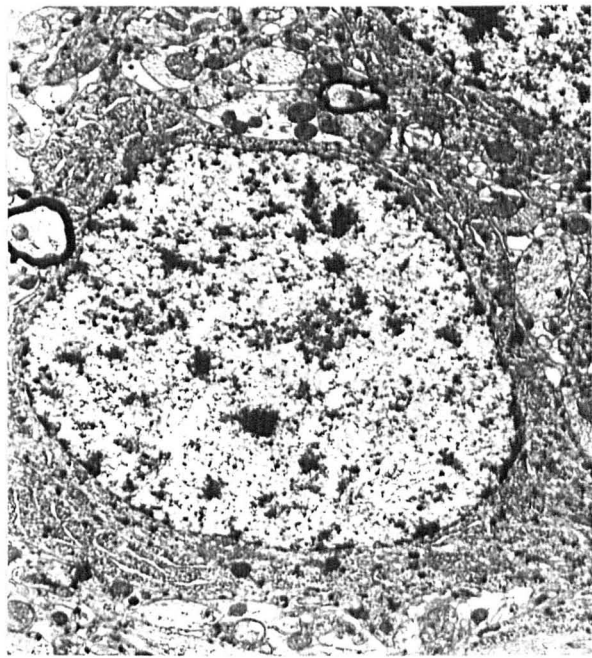


Figure 2.7: Electron micrograph of pyramidal cells of 7.5ml/min hippocampus (x6000).

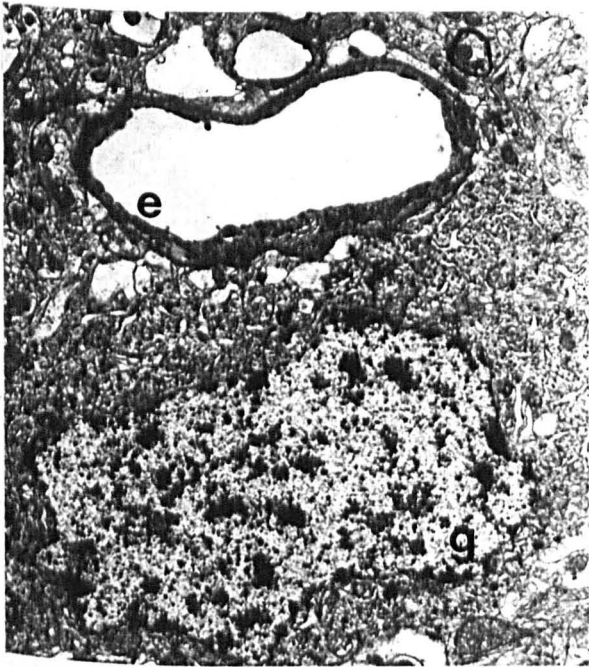


Figure 2.8: Electron micrograph of glial (g) and endothelial (e) cells from 7.5ml/min perfused brain (x6000)

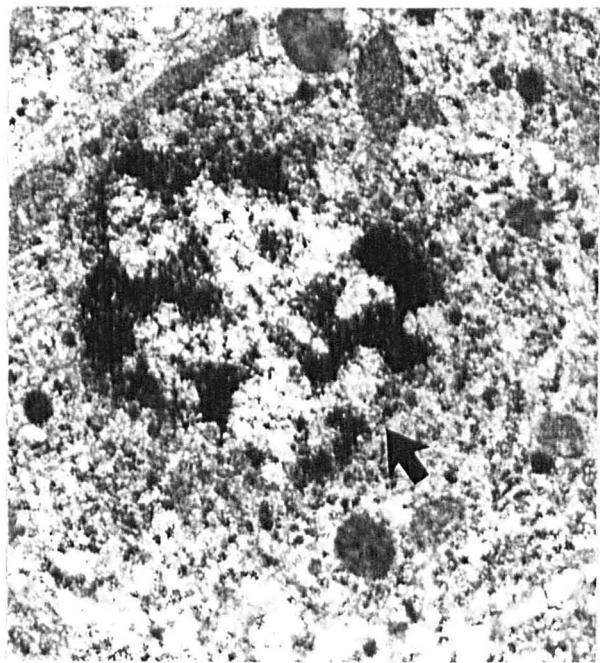


Figure 2.9: Electron micrograph of autolytic pyramidal cell from 5ml/min perfused brain. Note shrunken nucleus (arrow) (x20,000)

At a perfusion rate of 5mL/min, light microscopy showed good preservation of hippocampal cells. However, some shrinkage of cells was apparent and the perfusion rate was insufficient to clear out all blood cells from the vessels. In addition, the failure rate of perfusion with this flow rate was high (50%). Electron microscopy confirmed shrinkage of some pyramidal cells with some evidence of autolysis (Fig. 2.9).

On the basis of the results, a perfusion rate of 7.5 mL/min was selected as the optimum for preservation of hippocampal tissues.

Table 2.1: Assessment of fixation quality in rat hippocampus following perfusion fixation

Perfusion flow rate (mL/min)	Preservation of pyramidal cells	Perfusion artefacts present *	Number of animals used
22	-	yes	2
11	++	yes	2
9	+++	yes	2
7.5	+++	no	5
5	++	no	3

KEY

- = poor; + = moderate; ++ = good; +++ excellent

(defined by electron microscopy investigation)

* = artefacts such as dilation or rupture of membranes (high pressure) or shrinkage (low pressure)

2.3.3: Vascular visualisation method

The results obtained with the various dyes are summarised in Table 2.2.

Table 2.2: Results of perfusion of dye and fixative for brain vasculature

Dye used	Perfusion of dye in fixative	Perfusion of dye followed by immersion fixation
indian ink	++	+++
carmine	++	-
neutral fast red	++	-
methylene blue	-	-
permanent ink	-	-

Key: - few or no arteries and veins perfused
++ large arteries and veins perfused
+++ large and small arteries and veins perfused

Perfusion of dye with fixative produced acceptable results using indian ink, carmine and neutral fast red, particularly on whole brain preparations (Fig. 2.10). However, examination of 1mm thick brain slices showed that many minor arteries were not highlighted using this technique. The best results were seen when undiluted indian ink was perfused directly into the brain after prewashing. This highlighted many minor arteries and gave a better overall picture of the hippocampal vascular supply. It was clear that a number of the small transverse hippocampal arteries branched just below the pyramidal cell layer (Fig. 2.11). The vascular supply to CA1 region appeared poor when



Figure 2.10: Whole rat brain (x8) perfused with carmine in fixative (ventral aspect). Note vessels (arrows).

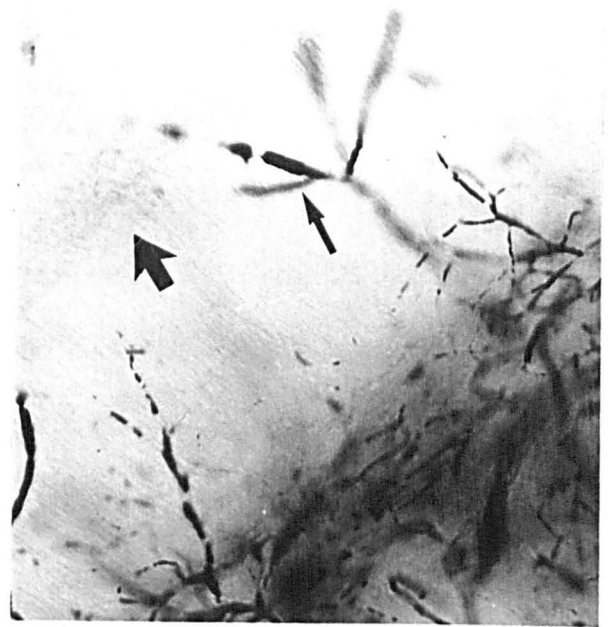


Figure 2.11: Indian ink perfused brain showing hippocampal cells (large arrow) and perfused vessels (small arrow) (x100)

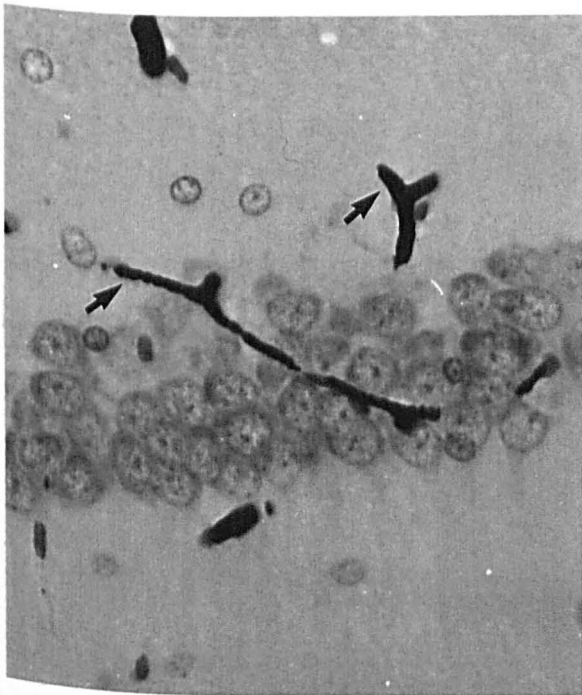


Figure 2.12: H&E section of hippocampus from an indian ink perfused brain (x500). Vessels are clearly visible by presence of ink (arrows).

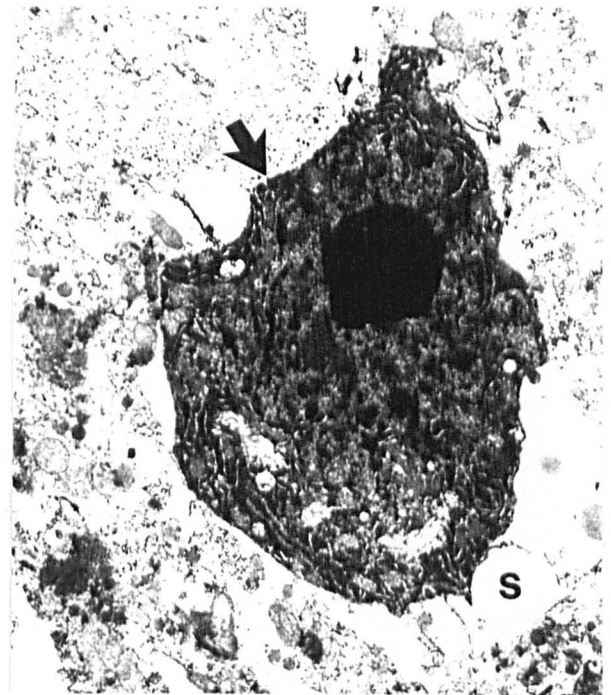


Figure 2.13:Electron micrograph of pyramidal cell layer showing a dark cell (arrow). Note shrinkage space (s) (x6000)

compared to other areas of the brain. Histological sections, when taken from tissue slices previously prepared for observation of vasculature, clearly showed dye present in the arteries and veins (Fig. 2.12)

2.3.4: Retrospective electron microscopy

Pyramidal cells were clearly visible in retrospectively examined tissue. Some cell bodies showed evidence of shrinkage, dense cytoplasm and pyknotic nuclei (Fig. 2.13) consistent with the artefactual "dark cell change" reported in immersion fixed hippocampus. There was also a degree of cellular disruption over and above that expected for immersion fixation. This was typified by mitochondria showing no cristae but containing amorphous material and some small circular granules. Endoplasmic reticulum was sparse and often artefactually dilated. Membranes were generally indistinct, profiles of myelin were present but difficult to observe in fine detail. Nerve cell processes were also indistinct. Endothelial cells were shrunken, with deformed contents. In general, tissue shrinkage was greater than that expected for immersion fixation in Karnovsky's fixative alone.

2.4 DISCUSSION

2.4.1: Perfusion fixation

Perfusion fixation has been used by many workers for the preservation of rat brain tissue. However, a variety of perfusion rates have been used and in some cases the rate is not specified. The effect of varying perfusion rate on the preservation of rat brain, particularly hippocampus, has not previously been reported. Rates of up to 40mL/minute have been used for perfusion fixation in the past [Auer et al 1984, Schmidt-Kastner & Hassman 1988]. These perfusion rates were used to study the effects of ischaemia and hypoglycaemia on the hippocampus and any abnormalities seen were attributed to treatment. However, these changes could be due to the high perfusion rates used since the micrographs show changes very similar to the perfusion-associated artefacts obtained with the higher flow rates used in the current project. In one case, no control animals were included in the study, making it impossible to compare the treatment-related effects with control animals perfused in the same way [Simon & Schmidley 1986].

The results described in this chapter indicate that the optimal perfusion rate for good preservation of the hippocampus with minimal perfusion artefact is 7.5mL/min, using the method detailed. Since this method has been validated using control animals, it is recommended that it be used in preference to immersion fixation for both light and electron microscopy in future studies involving potential hippocampal or other focal brain damage. A possibility for further evaluation in the brain would be to compare the results of perfusion pump apparatus with those using fixed head of pressure equipment. This was done in the subsequent work with the kidney. The resultant technique has subsequently been applied with success to the brain, and used in a recent toxicology study in the hamster which enabled the ultrastructural details of a specific effect in the brain to be studied (unpublished observation).

2.4.2: Vascular visualisation

Perfusion of the brain with fixative solution containing dye highlighted major arteries and veins in the hippocampus. Perfusion with dye alone followed by immersion fixation provided better results, picking up many of the minor arteries and veins as well as major ones. In all cases the most consistent results were obtained using indian ink as the dye solution. However, even here many capillaries were not filled when compared to results in the literature [Coyle 1976 & 1978].

Studies of whole brains and of 1mm-thick slices through the hippocampus region were used to build up a picture of the normal vascular pattern. The arterial supply to the hippocampus appears to be via transverse hippocampal arteries originating from the longitudinal hippocampal artery. This agrees with the vascular patterns described for the hippocampus by Coyle [1976 & 1978].

One of the great advantages of perfusing with dye and fixative is that it allows histological material to be processed from brain tissue previously prepared to display the vasculature, with very high quality results. When dye is perfused in conjunction with fixative, electron microscopy can also be performed. This technique could be used in future studies where hippocampal lesions occur, or in other tissues where the vasculature is implicated in the occurrence of a lesion. Observations from previous studies with hippocampal lesions show a degree of vascular alteration associated with the lesion area. Using this method it should be possible to visualise alterations and identify the vessels involved, and to identify whether the vasculature is primarily involved in the development of a lesion.

Compared to most areas of the brain, the number of dye-highlighted arteries and veins in the CA1 region of the hippocampus were few. This may reflect poor penetration of dye into the vasculature in this area or indicate that CA1 is inherently less well supplied than other areas. The latter

explanation has previously been put forward as the explanation for the susceptibility of the CA1 region to damage [Coyle 1978].

2.4.3: Retrospective electron microscopy

Although only one animal was studied, the results agree with those in the literature, namely that for detailed ultrastructural studies formalin fixed tissue provides very poor results. Many ultrastructural changes appeared to have occurred post-fixation, during storage in the fixative solution. This may be due to the build-up of formic acid which attacks the tissue [Bancroft & Stevens 1982]. The ultrastructural changes seen over and above those expected from immersion fixation alone are similar to those seen due to many pathological insults. Conclusions about the detailed pathology of the tissue would be very difficult. Furthermore, no information is available about the storage of tissue for longer periods (several years) and tissue damage may be increased over this period.

This method of observing "wet stored tissue" might be useful if only the assessment of the presence or absence of certain cell types is required. In addition, the presence or absence of specific intracellular granules such as lipofuscin might be possible. It has been reported that good ultrastructural preservation can be obtained if histological material is taken from wax block to the electron microscope level [Bancroft & Stevens 1982], providing a further means of retrospectively examining damaged tissues for electron microscopy. Processing wax-embedded tissue for EM should provide a better source of material since no further degradation of the tissue by the formation of formic acid (as occurs with tissue stored in formalin) will have taken place. This technique of processing wax-embedded material for electron microscopy has subsequently been evaluated and used with success in several toxicology studies where ultrastructural confirmation of observed histological changes was required.

2.4.4: Recommendations for Kidney work

The work carried out showed that perfusion fixation can successfully be used in toxicology studies for light and electron microscopic examination of brain tissue, including situations where ischaemic damage is suspected, or when tissues are particularly susceptible to fixation artefact, as is the case in the brain [Lamberts & Goldsmith 1986] and the renal papilla [Bohman 1974]. After optimising perfusion rates for evaluation of brain tissue using perfusion pump apparatus, the knowledge gained was applied to the kidney. Optimal fixation of kidney is preferable for light and electron microscopy, particularly in toxicology studies where it may be necessary to study subtle changes, such as vacuolation, without having to distinguish these from immersion fixation artefact. Both perfusion pump and constant head of pressure techniques were used and evaluated to determine which was preferable in terms of ease of use and reliability.

Perfusion with dye gave promising results for examination of the vasculature, especially when combined with clearing slices of tissue in cedarwood oil and subsequent histology. These techniques were developed and further assessed in the kidney work. Cedarwood oil was used for the examination of cleared slices of kidney, prior to histological assessment, to assess its use in aiding visualisation of vessels and damaged tubules. The technique of dye perfusion, and subsequent histology, was modified slightly by using a different dye (trypan blue) and assessing various routes of perfusion to evaluate this as a means of identifying necrotic cells in the kidney rather than highlighting alterations in the vasculature.

CHAPTER 3. BROMOETHANAMINE AND INDOMETHACIN STUDIES

3.1 INTRODUCTION

3.1.1: Experimental models of RPN

Although renal papillary necrosis (RPN) is one of the most common drug-associated nephropathies, there is still much discussion regarding the possible mechanisms of drug-induced RPN. Analgesic and non-steroidal anti-inflammatory drugs (NSAIDs) are capable of producing chronic RPN in both humans and animals, as discussed in the introductory chapter of this thesis, and laboratory animals treated with analgesics and NSAIDs have therefore been used to investigate the development and possible mechanisms. However, since laboratory animals are particularly sensitive to the extra-renal toxicity (e.g. gastrointestinal effects) and relatively resistant to the nephrotoxic effects of analgesics and some NSAIDs [Gregg et al 1989b, Wrenn et al 1991], the study of mechanisms of RPN using these compounds is difficult. Other experimental models of RPN have therefore been used with the aim of assisting in determining the events and mechanisms which lead to RPN in man. Two such experimental models are 2-bromoethanamine (BEA) and indomethacin, which can be used to induce extensive (BEA) or focal (indomethacin) RPN in an acute manner.

3.1.1.1: Bromoethanamine

2-Bromoethanamine (BEA; $\text{NH}_2\text{CH}_2\text{CH}_2\text{Br}$) is a low molecular weight, short chain aliphatic amine with pressor activity. It is readily soluble in water and is thought to rapidly undergo ring closure to a more stable compound [Bach et al 1980, Sabatini 1984]. BEA was first shown to cause experimental papillary necrosis in 1913 [Oka] and has since been shown in several investigations to induce extensive, reproducible RPN in all animals within 24-48h of a single intravenous or intraperitoneal dose of 50mg/kg or above [Bach et al 1980, Bach & Bridges

1982, Powell et al 1991]. The most striking functional changes seen following BEA treatment are polyuria (excess urine production) and an inability to concentrate the urine.

BEA is a well established experimental model of RPN and is considered a useful model for investigating the mechanistic basis of RPN and developing techniques to help identify RPN [Bach & Bridges 1982 & 1985, Sabatini 1984]. Investigations conducted using BEA as a model have tended to concentrated on identifying the course of lesion development with RPN and the primary cell types affected, and extrapolating the findings to RPN in man. Most of this work has been conducted in the rat, but pig [Gregg et al 1989b], hamster [Carlton & Engelhart 1989, Wolf et al 1992a], mouse [Gregg & Bach 1990, Wolf et al 1990, 1991b & 1992b] and gerbil [Wolf 1992a] have also been studied.

3.1.1.2: Indomethacin

Indomethacin is an NSAID which has been reported to induce RPN in combination with other analgesics in man [Jackson & Lawrence 1978, Clive & Stoff 1984]. It also causes focal RPN in rat following a single dose of 75mg/kg, within 48 hours of treatment [Wiseman & Reinert 1975, Molland 1978, Bach & Bridges 1982]. However, in contrast to BEA, the incidence of RPN induced with indomethacin is variable, and lesions can often be missed due to their focal nature [Arnold et al 1974, Bach & Bridges 1982]. As this is often also the case with RPN induced by analgesics and NSAIDS in man, indomethacin is a good model for studying possible mechanisms and in developing techniques to identify and study focal tissue damage. One limitation of using indomethacin (or other NSAIDS) to induce experimental RPN is that it also induces gastrointestinal lesions in rats 48 hours after treatment of a single dose [Arnold et al 1974, Clive & Stoff 1984], so it cannot be used in larger doses or for longer dosing periods.

3.1.2: Development of renal papillary necrosis with BEA and indomethacin

The pathological changes in the papilla produced with BEA are similar to those seen with analgesic nephropathy RPN in man and have been described in increasing detail [Hill et al 1972, Murray et al 1972, Cuttino et al 1981, Bach et al 1983, Sabatini 1984, Gregg et al 1990a & b, Gregg & Bach 1990, Wolf & Carlton 1990]. It was originally suggested that the first effects are seen in the capillaries [Hill et al 1972], but it has since been proposed, following detailed time-course studies in rodents, that the earliest effects are seen in the renal medullary interstitial cells [Mattingley et al 1984, Bach & Bridges 1985, Bach & Hardy 1985, Bach et al 1991, Gregg et al 1990 a & b, Gregg & Bach 1990]. These show slight changes at 4 hours post-dose (pyknotic or irregular nuclei and increased mucopolysaccharide staining), with focal necrosis apparent at 8 hours. Endothelial degeneration with platelet adhesion, and denudation of the covering epithelium of the papilla tip are also seen at 8 hours post dose. By 12 hours the interstitial cells show marked necrosis. The platelet adhesion is marked at 18 hours post-dose, but only in the affected capillaries. Extensive degenerative changes within the papilla are seen at 24 hours post-dose with a greater area of the papilla affected.

The pathological changes leading to RPN with indomethacin treatment have not been studied in as much detail as BEA, but the sequence of events is similar. The major difference, as mentioned earlier, is that RPN induced with indomethacin is focal in nature, and therefore the extensive RPN reported with BEA in the later stages is not seen with indomethacin.

3.1.3: Possible mechanisms of experimental renal papillary necrosis

Despite much research into the pathological changes induced in the papilla with BEA and indomethacin, the mechanism by which this RPN develops is still unknown [Bach & Bridges

1985, Bach 1991, Wolf 1992b]. The possible mechanisms proposed mirror those suggested for RPN in man and can be summarised as follows: i) counter-current concentration mechanism; ii) immunotoxic effects of the compound; iii) perturbation of renal intermediary metabolism; iv) direct or indirect renal ischaemia and vasoconstriction; v) generation of biologically reactive intermediates leading to excess lipid peroxidation and cellular injury. The two most popular mechanisms quoted are renal ischaemia and generation of reactive intermediates.

Vasoconstriction has been proposed as the mechanism of RPN with BEA by several investigators [Cuttino et al 1981, Wyllie et al 1972, Russell et al 1987, Wolf et al 1991b & 1992a], but this suggestion has been disputed by others [Solez et al 1974, Sabatini 1984, Bach & Bridges 1984]. It has also been suggested that the most likely mechanism involved with BEA-induced RPN is the generation of biologically reactive intermediates, leading to effects on peroxidative activity and lipid peroxidation in the interstitial cells. This would then initiate a cascade of events leading ultimately to RPN [Bach et al 1986]. Supporting this is the suggestion that conditions within the papilla (such as the renal concentrating mechanism) may favour the concentration and subsequent dissociation of BEA into reactive alkylating species [Powell et al 1991].

Indomethacin is known to inhibit the vasodilatory prostaglandin PGE_2 , which is produced mainly in the renal medullary interstitial cells and has a local action [Arnold et al 1974, Jackson & Lawrence 1978, Melendez & Reyes 1982, Zenser & Davies 1984, Zenser et al 1985]. It has therefore been proposed that this effect is involved in the development of RPN with indomethacin via alterations in renal haemodynamics leading to renal ischaemia [Jackson & Lawrence 1978, Henrich et al 1978, Bach & Bridges 1984, Heyman et al 1988 & 1991].

However, indomethacin also has an inhibitory effect on prostaglandin synthetase, lipooxygenase and cyclo-oxygenase, present within the interstitial cells, so the mechanism of RPN with indomethacin may be via an effect on the interstitial cells with generation of reactive species, and subsequent cascade effects [Bach & Bridges 1984, Marnett et al 1984, Kirkova et al 1992 & 1995].

Whatever the mechanisms responsible for the development of RPN, and there may be several, there is still much discussion and research involved in detecting the lesion at an early stage and elucidating the mechanistic basis of this lesion. This will be useful for the identification and subsequent management of RPN in man, and in the design of new drugs which do not induce such effects.

3.1.4: Detection and identification of RPN

Experimental models of RPN are not always reproducible (as is the case with indomethacin), and the analytical techniques used may lack sensitivity so that identifying the events prior to, or in the early stages of, necrosis is very difficult. At present, accurate diagnosis of the presence of focal RPN relies on the examination of serial histological sections which is very time-consuming [Arnold et al 1974, Bach & Bridges 1982 & 1985]. Any development of techniques which help in the diagnosis of RPN would therefore potentially be very useful, particularly development of reliable non-invasive markers for nephrotoxicity. These could be used to screen for the presence of lesions in animals and humans, ideally in the early stages of lesion development when recovery is still possible [Zbinden et al 1988, Price et al 1996].

3.1.4.1: Potential methods for investigation

Urine is the major body fluid for the noninvasive investigation of damage and dysfunction to

the kidney [Stonard et al 1987]. Urinary sediments (cytology) have been used with some success in man and in animal studies to investigate and help indicate the presence of various types of kidney damage, but have often been compared with urinary enzyme levels rather than with histopathology findings. They have been used in man for the diagnosis of transplant rejection [Madras et al 1986], assessment of inflammatory state [Guthinger et al 1977], detection of acute renal failure [Gay et al 1978, Mandal et al 1985, Mandal 1988, Mandal & Bennett 1988], aminoglycoside nephropathy [Coulon et al 1984], and interstitial nephritis [Pitone et al 1982, Spence et al 1985].

Sediments were found to show a good correlation with pathological data in acute renal failure in children [Gay et al 1978]. Mandal and co-workers [1985, 1988, Mandal & Bennett 1988] investigated the usefulness of urinary sediments (using transmission electron microscopy) in detecting acute renal failure in patients with acute tubular nephropathy and aminoglycoside damage (both cortical effects). They found a good correlation between the type of sediment and the severity of the illness, and could distinguish between different cell types in the sediment. They also emphasised an important point when assessing urinary sediments; that the usefulness of sediments depends upon adequate collection and preparation methods. Coulon et al [1984] also found sediments useful for detecting aminoglycoside nephropathy and recommended their use as a non-invasive marker. Spence et al [1985] used sediments to look for urinary crystals and casts in triamterene-treated patients (who can develop interstitial nephritis), and found them useful for showing a treatment-related brown precipitate in the urine.

Jackson et al [1978] studied urine cytology in analgesic nephropathy with regard to carcinoma screening and recommended its use as a screen for possible carcinoma or dysplasia in analgesic abusers with RPN (i.e. those patients at risk of developing urothelial malignancies). Russ et al

[1982] also used urine cytology for the same purpose. Jackson and co-workers [1978] found that more severe cytology changes were observed in cases of more severe renal failure and suggested that urinary sediments might therefore also be useful to help detect RPN in patients.

Urine sediments have been investigated in animal studies to indicate renal damage following treatment with the cortical toxins 2,2,4-trimethylpentane [Fowlie et al 1987] and mercuric chloride [Kennedy & Gail Saliya 1970], and the papillotoxins BEA and aspirin [Davies et al 1968, Kennedy & Gail Saliya 1970]. A non-invasive nephrotoxicity screening test based on repetitive, quantitative urine analysis in rats has been proposed as a useful procedure to detect nephrotoxic chemicals acting by a variety of mechanisms [Fent et al 1988, Zbinden et al 1988]. Fent and Zbinden and co-workers [1988] looked at 17 reference substances, including the analgesics phenacetin and phenylbutazone, using female Sprague-Dawley rats. They studied the effects in the urine (volume, pH, enzymes, proteins, cells present) following treatment and then compared these with histology data. Elevated excretion of cells and occurrence of cylinders were considered the most sensitive indicators of renal damage. The findings from these various studies suggest that urinary sediments may therefore be of potential value in the early detection of necrosis.

Trypan blue uptake is routinely used to assess the viability of cultured cells (it is excluded by living cells and retained in necrotic cells) and has also been used to investigate zonal hepatotoxicity in perfused livers with good results [Belinsky et al 1984]. It may therefore be useful in identifying areas of necrosis in the kidney, particularly in focal damage which may be missed in routine examination.

Scanning electron microscopy (SEM) has been used successfully for the investigation of

bacterial infection associated with RPN [Cohen et al 1979 & 1981] and for studying morphological changes in bladder epithelium following acute toxicity [Okamura et al 1992] or treatment with proliferative agents [Shibata et al 1989 & 1991, Anderson 1991]. However, no work has been reported using SEM for the investigation and detection of RPN per se. SEM may be of use as a large area of tissue can be examined at once, allowing examination of the whole papilla rather than a thin section through this area. It should also be possible to produce histological sections from SEM material, to allow comparison of the appearance of a lesion with the two techniques.

3.1.5: Aims of the studies described in this chapter

The aims of the work detailed in this chapter were to evaluate the methods mentioned above for their usefulness in identifying extensive and focal lesions in the kidney, induced using BEA and indomethacin respectively. Also, initial investigations were performed to assess the accuracy of the standard technique used to identify RPN, a histological section through the papilla tip [Arnold et al 1974, Bach & Bridges 1982 & 1985), using both BEA and indomethacin.

The usefulness of urinary sediments, when compared with histology, in determining RPN 24 and 48 hours after treatment with BEA and indomethacin was investigated. Also, the use of cedarwood oil to clear kidneys during histological processing, for observation of vasculature and areas of necrosis within thick slices of tissue, was evaluated for its potential to help identify RPN. This had proven useful in allowing visualisation of the vasculature in the preliminary work carried out with the brain (Chapter 2), particularly when combined with dye perfusion techniques.

The dye perfusion method developed with the brain for visualisation of the vasculature was used for a different purpose in the kidney; it was applied to assess the usefulness of trypan blue perfusion for highlighting areas of necrosis in both extensive and focal RPN caused by BEA and indomethacin respectively. Different routes of perfusion were assessed to find the best means of getting dye into the kidney structures, particularly the papilla. The technique of clearing in cedarwood oil during histological processing to help visualise any dye present, which had been used successfully in the brain, was also performed with un-perfused and trypan blue-perfused kidney slices to determine whether this was also useful for identifying necrotic areas in the kidney.

SEM was included in the investigations to assess its potential usefulness for identifying the morphological changes associated with RPN and studying its effects. The feasibility of subsequently processing SEM material for correlative histology, and the quality and usefulness of the results obtained, was also assessed. The same principle of performing "secondary" histology to gain more information had worked well in the brain for examining brain slices which had previously been perfused with dye, cleared with cedarwood oil, and evaluated by light microscopy.

The ultimate aim of this chapter was to identify the techniques which may be of use in identifying RPN, to enable further assessment of these techniques in the subsequent chapter with a potential new model for RPN, the antioxidant ethoxyquin. Comparisons could then subsequently be made between the usefulness of the techniques, the effects of the three models (BEA, indomethacin and ethoxyquin), and the potential mechanisms involved in the development of RPN.

3.2 MATERIALS AND METHODS

3.2.1: Materials

3.2.1.1: Chemicals and suppliers

All buffer salts, formaldehyde (40% stock), alcohol, acetone, xylene, cedarwood oil and paraffin wax were obtained from BDH Merck Ltd. Neutral buffered formalin (10%) and all histological dyes used in the Haematoxylin & Eosin and Papanicolaou staining methods were obtained from Pioneer Research Chemicals. Shandon Cytospin fluid was obtained from Life Sciences International, Runcorn, Cheshire. Trypan Blue was obtained from Sigma Aldrich, Poole, Dorset.

Electron microscopy fixatives, chemicals, and specimen stubs were obtained from Agar Scientific Ltd, Essex. "Sagatal" anaesthetic was obtained from Rhone-Merieux. Heparin ("Monoparin") and local anaesthetic ("Lignavet"), used in perfusion experiments, were obtained from CP Pharmaceuticals Ltd., and C-Vet Ltd. respectively. Bromoethanamine was obtained from Sigma Aldrich. Indomethacin was manufactured and supplied in-house. Gum tragacanth (vehicle for indomethacin) was obtained from Red Carnation Gum Co., Sir John Lyon House, London.

3.2.1.2: Animals, housing conditions and dosing regimes

Female Sprague-Dawley rats from Charles River (UK) Ltd., Margate, Kent, each weighing 150-200g, were used in all experiments conducted with bromoethanamine (BEA) and indomethacin. Animals were housed at a temperature of $21 \pm 2^{\circ}\text{C}$, relative humidity of $55 \pm 10\%$ and 12 hours light/12 hours dark lighting. Animals were fed with a standard certified rodent diet (RMM diet, Special Diet Services, Cambs., UK), both this and water were provided *ad libitum*. The practical work carried out in this chapter was separated over several experiments for logistical reasons; the numbers of animals used in each experiment are detailed in Table 3.1.

Table 3.1: Number of animals used in BEA and indomethacin experiments and numbers used for each technique

Technique	BEA			Total Numbers	indomethacin			Total Numbers
Histology	20 (20)	6 (3)	10 (5)	36 (28)	20 (20)	6 (3)	10 (5)	36 (28)
Serial sections	4 (4)	-	-	4 (4)	10 (0)	-	-	10 (0)
Cedarwood oil	10 (10)	6 (3)	-	16 (13)	10 (10)	6 (3)	-	16 (13)
Urine sediments	20 (20)	-	10 (5)	30 (25)	20 (20)	-	10 (5)	30 (25)
Urine volumes noted	No	No	Yes	10 (5)	No	No	Yes	10 (5)
Trypan blue	-	6 (3)	10 (5)	16 (8)	-	6 (3)	10 (5)	16 (8)
SEM	-	6 (3)	10 (5)	16 (8)	-	6 (3)	10 (5)	16 (8)
Secondary histology	No	Yes	Yes	16 (8)	No	Yes	Yes	16 (8)
EXPERIMENT	A	C	D	-	B	C	D	-

The number of treated animals used for each technique is given first, the number of control animals is given in brackets. The numbers used are split into columns for each separate experiment conducted. Experiments are coded A-D at the bottom of the table.

The dosing regime for BEA-treated animals was a single intravenous injection via the tail vein at a dose of 150 mg/kg in 0.9% sodium chloride; indomethacin-treated animals were given a single oral gavage (oral intubation) dose of 75 mg/kg in 25% gum tragacanth. The control animals for each treatment compound were dosed with the appropriate vehicle by the same route. Unless otherwise specified, all animals were killed 24 hours after the last dosing by an intra-peritoneal overdose of sodium pentobarbitone ("Sagatal", 0.2mL/100g body weight).

3.2.2: Histology

Animals were killed as outlined in 3.2.1.2, either 24 or 48 hours post-dose. Both kidneys were removed and immerse-fixed separately in 10% neutral buffered formalin for at least 2 days. Thin slices (<1mm) through the centre of each kidney (containing the papilla) from 10 BEA-treated and 10 indomethacin-treated animals were cut by hand using skin graft blades, dehydrated by hand through a graded alcohol series (25, 50, 75, 90 and 2 x 100% ethanol, 30 minutes each), and cleared in cedarwood oil for 48 hours to assess the use of this technique in aiding visualisation of vasculature and damaged tubules. Macroscopical and microscopical assessment of the cleared slices was carried out. After assessment of the cleared kidneys, the slices were infiltrated with paraffin wax for 48 hours in an oven at 58°C and embedded in paraffin wax blocks using a Tissue-Tek embedding centre.

Slices through the centre of each kidney from the remaining animals were processed through to paraffin using a Bayer VIP automated tissue processor (50, 80, 95, and 2 x 100% ethanol, 3 x xylene, 1 hour each at 40°C; paraffin wax 3 x 2 hours at 60°C), using the standard clearing agent, xylene, instead of cedarwood oil. Embedding was carried out as above. Sections through the centre of each papilla were cut at a thickness of 4µm using an Anglia Scientific microtome. Sections were stained with Haematoxylin & Eosin (H&E, method in Appendix 1) on a Shandon Varistain staining

machine for histological assessment. The definition of kidney necrosis in each technique and the basis for categorisation was as follows:

Cedarwood oil: Alterations in vasculature (condensed & reddened areas) and pale areas of papilla were recorded as abnormalities.

Histology: The grading system used for assessment of renal papillary necrosis (RPN) is detailed in Table 3.2 below.

Table 3.2: Grading criteria for histological and electron microscopy assessment of renal papillary necrosis

Grade	Histological appearance
Minimal	slight damage to papilla tip with necrosis of interstitial cells and some epithelial cells in the loops of Henle, preservation of all ducts & tubules
Moderate	loss of interstitial cells, derangement and necrosis of some tubules (mainly loops of Henle) but preservation of papilla structure, hyaline granules present in some collecting duct cells
Marked	destruction of papilla structure within lesion; loss of interstitium, tubules and ducts (outline only remaining). In BEA-treated animals, the outer papilla shows loss of interstitium, with derangement of some loops of Henle and capillary endothelium. Hyaline granules in collecting duct cells in less damaged areas.

3.2.2.1; Additional Histology

BEA-treated animals:

Re-examination

The initial assessment of BEA-treated kidneys as detailed in 3.2.2 above raised a question regarding whether one kidney from an animal was a reasonable predictor of the other in terms of degree of necrosis. To answer this question, kidneys were randomised for re-examination and the degree of

necrosis in each kidney graded in terms of “% damage”, that is, the percentage of the length of each papilla with damaged structures (rather than complete necrosis); this was to give a “quantitative” aspect to the extent of damage to the kidney and allow statistical analysis. Following this re-examination, the kidneys were un-randomised and the gradings assessed to determine how close the kidneys from one animal were in terms of % damage. Statistical analyses (scatterplots and correlation coefficients) were performed to assist in this assessment.

Step-sections

Examination of histological sections as detailed in 3.2.2 identified some animals where the kidneys had not been sectioned through the actual papilla tip (no Ducts of Bellini opening out of papilla). The grading of these kidneys was also less severe. Further work was carried out in an attempt to determine how much the distance from the tip affected the extent of necrosis.

Slices through the middle of the kidney were taken from both kidneys from two BEA-treated animals and processed separately to paraffin blocks using a Bayer VIP automated tissue processor as previously described. Sections were cut and collected every 20 microns through the whole thickness of the papilla from each kidney. These were then stained with H&E for assessment as described above.

Gradings of RPN were given a score of 0 - 3 to assist in the recording of results, and the relative position of each section within the papilla was assessed and graded as detailed in Table 3.3. The aim of this was to determine the amount of variation in damage throughout the papilla and subsequently whether the extent of damage was consistent with the position within the kidney (i.e. whether the damage was worse nearest the tip).

Table 3.3: Details of system used for scoring of necrosis and section position

Score	Definition	Position	Definition
0	Normal	1	section through tip, showing openings of ducts of Bellini
1	Minimal necrosis	2	section through tip, but ducts of Bellini not present in section
2	Moderate necrosis	3	section near to tip but not through
3	Marked necrosis	4	section well away from tip

Indomethacin-treated animals:

Step sections

Both kidneys from all treated animals killed at 24 hours were sectioned further after the initial histological assessment to determine the likelihood of missing focal lesions if sections are only taken through the papilla tip. All kidneys were sectioned through to the end of the block; every third section was collected on slides and H&E sections prepared (resulting in up to 40 extra sections per block, 80 per animal). These "step serial" sections were then examined for presence of lesions.

3.2.3: Urinary sediments

Urine samples were collected into ice-cooled collecting vessels 0-8, 8-24 and 24-48 hours post-dose with BEA or indomethacin. Animals were housed in MS&D (modular systems & development) metabolism cages for urine collection. Aliquots of urine (0.25ml) were mixed with an equal volume of Cytospin fluid (to fix cells in the urine) and urinary sediments were prepared from these by centrifugation using a Shandon Cytospin according to the manufacturer's instructions (20 minutes at 1500 r.p.m). Sediments were stained prior to assessment with the Papanicolaou histological stain, using a Shandon Varistain staining machine (method as detailed in Appendix 1), and the sections assessed by light microscopy to determine types of cells present and numbers of

transitional epithelial and renal tubule cells were present. An increase in the number of epithelial or transitional cells compared to controls and the presence of renal tubule cells was taken as an indication of renal necrosis.

In later experiments, urine samples were collected into ice-cooled collecting vessels 0-24 hours post-dose as above and urine volumes were measured. Fixed urinary sediments were prepared as above. The number of transitional cells present in the sediment (including renal tubule cells) were noted microscopically by manually counting the number of cells in each x200 magnification field, for the whole sediment area. Cell numbers were then converted to give total cells present in the urine based on the volume of urine collected as follows:

total cells = cell numbers in sediment x 4* x volume of urine collected (mL)

(*aliquot of 0.25mL corrected to 1mL)

Statistical analysis of the results (analysis of variance) was performed to determine the significance of any differences in cell numbers.

3.2.4: Perfusion experiments

Animals were killed as outlined in 3.2.1.2. Under deep Sagatal anaesthesia, kidneys were perfused by one of the methods below (perfusion pump or fixed head of pressure). After perfusion, kidneys were removed and processed to paraffin block using a Bayer VIP processor (processing cycle as in 3.2.2) and H&E sections produced for histological assessment as detailed in 3.2.2.

3.2.4.1: Perfusion pump apparatus - local perfusion

The abdomen was opened and the gastrointestinal tract gently pushed to the right hand side. The abdominal aorta was located and tied off with suture thread above the kidney as close to the aortic

branch as possible (limiting perfusion to the kidneys only). The aorta below the kidney was then lifted with curved forceps and thread placed underneath. A cannula derived from a 25 gauge needle was inserted into the aorta and tied in place using the thread. The cannula was attached to a 60mL syringe containing a prewash solution of 0.9% saline containing heparin (25 units/mL) and "Lignavet" local anaesthetic (0.4mg/mL). The syringe was placed in a Wellmed syringe pump. Once the cannula was in place and tied, the abdominal vena cava was cut and the pump started.

Various flow rates were used to compare their effects on preservation and morphology of the papilla; the rates used were 6, 10, 20 and 25 mL/minute. After 50mL of the prewash had been pumped through the animal, flushing out the blood, the cannula was clamped and the syringe on the pump changed for one containing Karnovsky's fixative (2.5% glutaraldehyde and 2% formaldehyde in 0.1M phosphate buffer pH 7.4). The pump was restarted and the clamp removed, allowing fixative to perfuse through the kidney. After 30mL of fixative had perfused through, the kidneys were fairly rigid.

3.2.4.2: Fixed head perfusion apparatus - whole body perfusion

Fixed head perfusion apparatus was set up as shown in Fig 3.1. The solutions used for perfusion were the same as those used for 3.2.4.1 above. A sphygmomanometer was used to monitor the perfusion pressure in mm of mercury (mm Hg) and a hand pump from the manometer used to maintain the pressure. The perfusion pressure used was 110 mm Hg.

Under terminal anaesthesia, the thoracic cavity of the animals was opened to reveal the heart and associated vessels and the rib cage deflected to allow access. The right ventricle of the heart was gripped with toothed forceps and the apex of the heart snipped to give access to the left ventricle. A rat gavage cannula was inserted through the left ventricle into the aorta and clamped in place

with artery forceps (just above cut in ventricle). The inferior vena cava beneath the heart was immediately cut and the saline flow started. When all the organs had cleared of blood, flow was switched from saline to Karnovsky's fixative. After 3-5 minutes, when approximately 400ml of fixative had gone through, tissues were firm and the animal was rigid. Kidneys were left in situ for 10-15 mins before removal to allow the fixative to penetrate the tissues further.

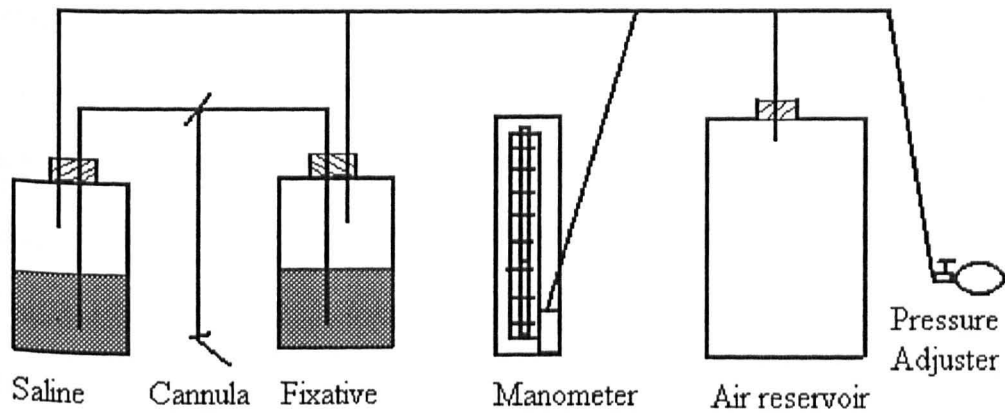


Figure 3.1: Fixed head perfusion apparatus

3.2.5: Trypan blue perfusions

3.2.5.1; Assessment of perfusion route

Prior to using trypan blue with treated animals, 3 control animals were perfused with trypan blue, one by each of 3 routes to determine the best means of ensuring trypan blue entered the kidneys. The routes assessed were: whole body perfusion; local perfusion via the abdominal aorta and retrograde perfusion via the ureter. The first 2 methods of perfusion were carried out as detailed in 3.2.4 above except that 0.2mM trypan blue solution was perfused instead of saline or fixative, ureteral perfusion was performed in a similar manner to local perfusion via the aorta, except that the cannula was inserted into the ureter below the kidney.

3.2.5.2: Perfusion experiments

Twenty four hours post-dose, control, indomethacin- and BEA-treated animals were given an intraperitoneal injection of pentobarbitone (Sagatal, 0.2 mL/100g). Under deep anaesthesia, the kidneys of all animals were locally perfused via the abdominal aorta using perfusion pump apparatus set at 20 mL/minute flow rate (as determined in 3.2.4 and 3.2.5.1). Kidneys were perfused with 0.9% saline containing heparin (25 units/mL) and "Lignavet" (0.4mg/mL) until cleared of blood and then for 5 minutes with 0.2mM trypan blue. The right kidney was tied off with suture and the left kidney perfused with 0.9% saline to remove excess trypan blue for comparison. Both kidneys were then removed and immerse-fixed in 10% neutral buffered formalin for at least 2 days.

Kidneys were processed through to paraffin block, some samples were processed by hand as described earlier (3.2.2) to enable them to be examined after "clearing" in cedarwood oil, the rest were processed using a Bayer V.I.P. tissue processor and xylene instead of cedarwood oil as clearing agent (as 3.2.2). Both H&E and eosin-only stained sections (to help identify any trypan blue present) were produced for histological assessment, using the criteria outlined in 3.2.2.

3.2.6: Scanning electron microscopy (SEM)

Kidneys from control, indomethacin- and BEA-treated animals (48 hours post-dose) were processed for SEM investigation after perfusion or immersion fixation. Samples were washed in distilled water to remove any buffer salts from the surface of the tissue, then dehydrated through a graded series of acetone (25, 50 and 75% acetone 20 mins each, 100% acetone overnight). Samples were then critical point dried for a minimum of 1 hour to remove any liquid from the tissues and replace it with liquid CO₂. Critical point drying was carried out using a Polaron critical point dryer; this process replaces any liquid in the tissue with liquid CO₂. Liquid CO₂ instantly

sublimes to gas at its "critical point" (36°C or 1200 p.s.i. pressure), leaving dry specimens with optimal preservation and minimal shrinkage. Samples were sputter coated with gold in a Polaron SC500 sputter coater; coating the specimen with a thin layer of gold conducts electrons and prevents "charging" (accumulation of electrons on the surface which distorts the image).

Specimens were mounted on aluminium stubs with double-sided tape and examined in a Philips XL20 SEM operating at 10kV, at magnifications from 20 to 10,000 times. Both the internal and surface structures of the papillae were assessed for any signs of derangement or necrosis.

3.2.7: Correlation of SEM and histology

Following SEM assessment, papilla samples were removed from the specimen stubs, placed in tissue cassettes and processed to paraffin wax using a Bayer VIP automated tissue processor as detailed in 3.2.2. Samples were embedded in paraffin wax blocks using a Tissue-Tek embedding centre. Sections (4µm-thick) were then cut on an Anglia Scientific microtome and stained with H&E for histological assessment (both of tissue preservation and treatment effects) and to allow correlation with the SEM findings from the same samples.

3.2.8: Statistical analysis

Scatterplots and correlation coefficients were used to determine the significance of results in re-examination of the experimental animals in section 3.2.2.1.

**Table 3.4: Summary of histological findings: incidence and grading of renal papillary
necrosis**

	24 hours						48 hours					
	control		BEA		indomethacin		control		BEA		indomethacin	
	R	L	R	L	R	L	R	L	R	L	R	L
Cedarwood oil:												
NAD	10	10	6	6	10	10	10	10	6	4	0	0
Possible necrosis*	0	0	4	4	0	0	0	0	4	6	0	0
Total animals	10		10		10		10		10		10	
Histology:												
NAD	20	20	0	0	8	10	19	20	0	0	8	7
Mild necrosis	0	0	3	3	1	0	1	0	1	4	0	1
Moderate necrosis	0	0	6	3	0	0	0	0	6	2	1	2
Marked necrosis	0	0	1	4	1	0	0	0	3	4	1	0
Total animals	20		10		10		20		10		10	

KEY

BEA = Bromoethanamine R = right kidney; L = left kidney

* = Necrosis interpreted as alterations in vasculature (congested & reddened areas), pale areas in kidney.

NAD = no abnormalities detected.

Numbers in table are number of animals affected. Total number of animals assessed for treatment group are also detailed in table.

3.3 RESULTS

3.3.1: Histology

All BEA-treated animals showed signs of necrosis varying from mild to marked renal papillary necrosis (RPN) on histological examination at both 24 and 48 hours (Figs 3.2a and 3.2b); mild necrosis affected only interstitial cells and some epithelial cells of loops of Henle, moderate necrosis affected interstitial cells and necrosis of some tubules, marked necrosis was typified by destruction of the papilla structure within the area of the lesion. Necrosis was bilateral in all BEA-treated animals, although the severity of necrosis varied between kidneys, as shown in Table 3.4. One notable feature in BEA-treated animals 48 hours after treatment was the presence of mitotic figures in collecting duct cells (and occasionally in preserved loops of Henle). No mitotic figures were seen in either control animals, indomethacin-treated animals, or in BEA-treated animals 24 hours after treatment.

Assessment of the cedarwood oil-cleared slices at 24 and 48 hours showed reddening of the papilla due to congestion, suggesting renal damage in 10 of the 20 BEA-treated animals where this step was carried out. No necrosis was apparent in the cedarwood oil slices from indomethacin-treated animals. Histological examination of kidney sections from indomethacin-treated animals from both time points identified 5 treated animals with signs of necrosis of varying degree, ranging from mild to marked as described above for BEA lesions (Fig 3.3). Two of these had bilateral necrosis (Table 3.4).

Figure 3.2a: Grades of papillary necrosis induced with BEA- low power micrographs



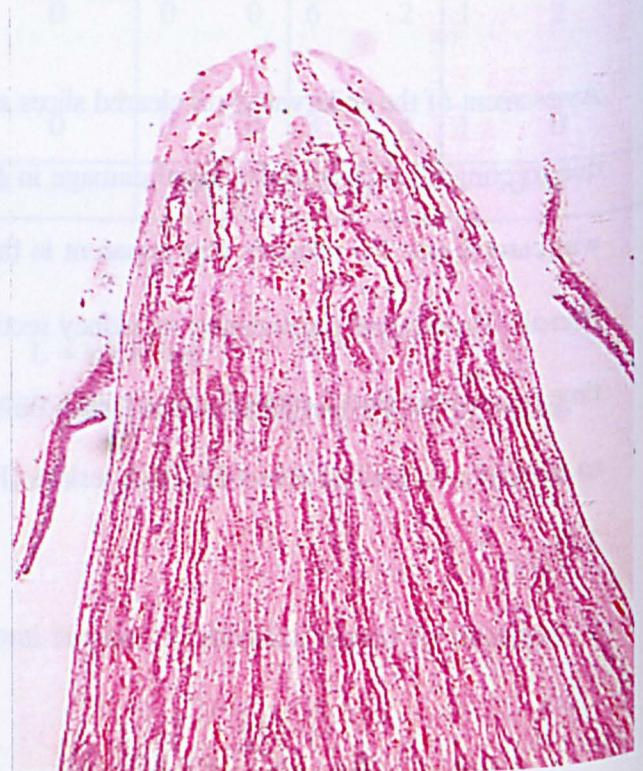
Normal papilla: magnification x 50.



Mild necrosis: note loss of interstitial detail (arrow). Magnification x 50.

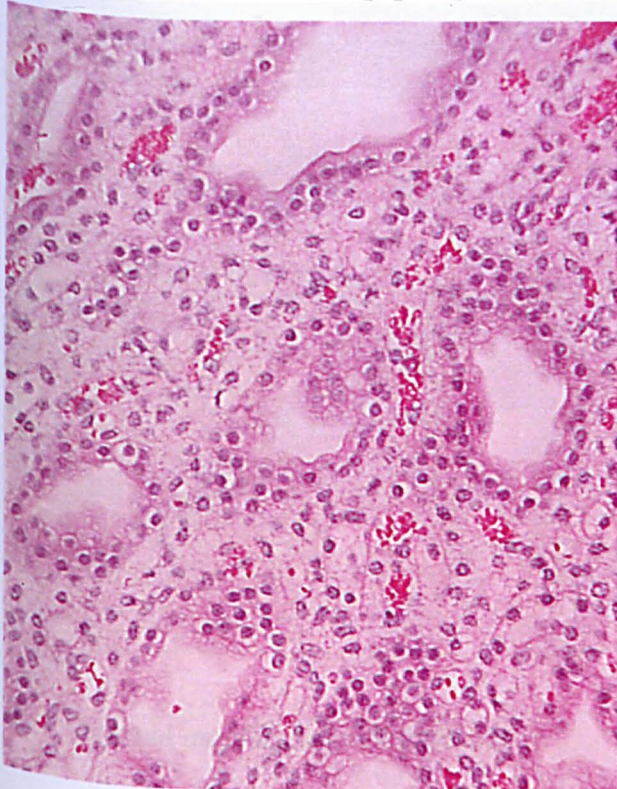


Moderate necrosis: Note loss of interstitial cells; degeneration of tubules in tip region but outline of structures still visible (arrow). Magnification x 50.

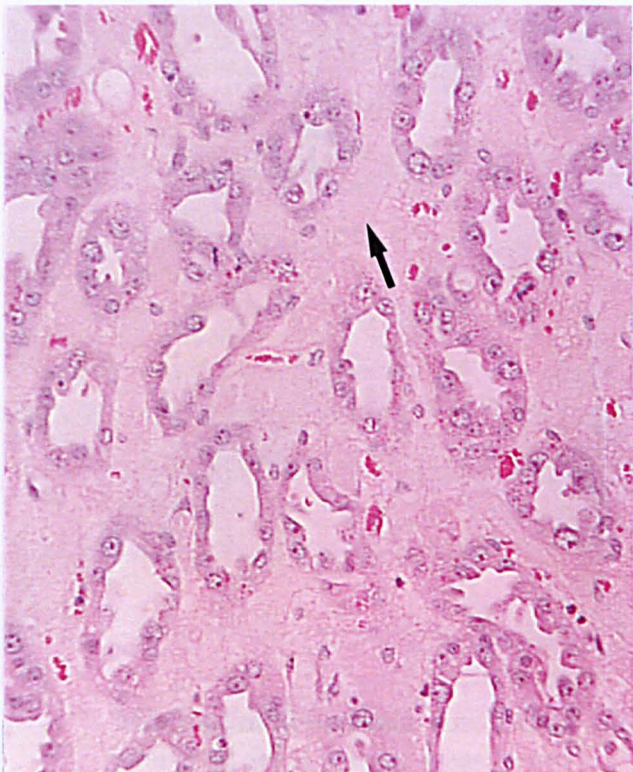


Marked necrosis: Loss of all structural detail in papilla tip area. Magnification x 50.

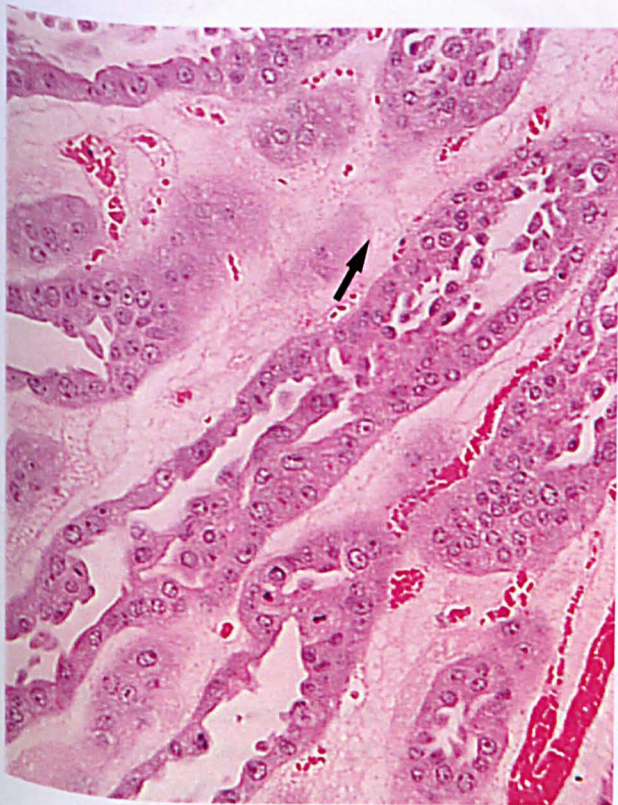
Figure 3.2b: Grades of papillary necrosis induced with BEA- high power micrographs



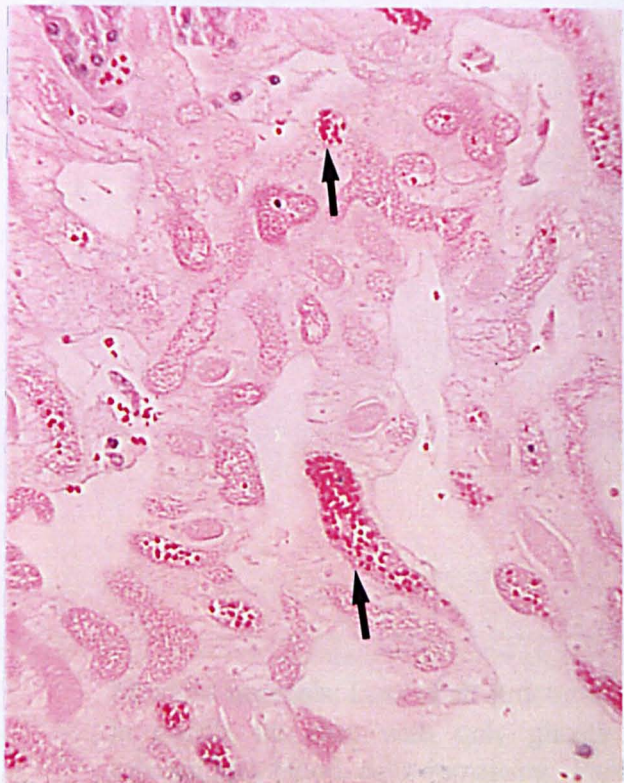
Normal papilla: magnification x 250.



Mild necrosis: note loss of interstitial cells (arrow). Magnification x 250.

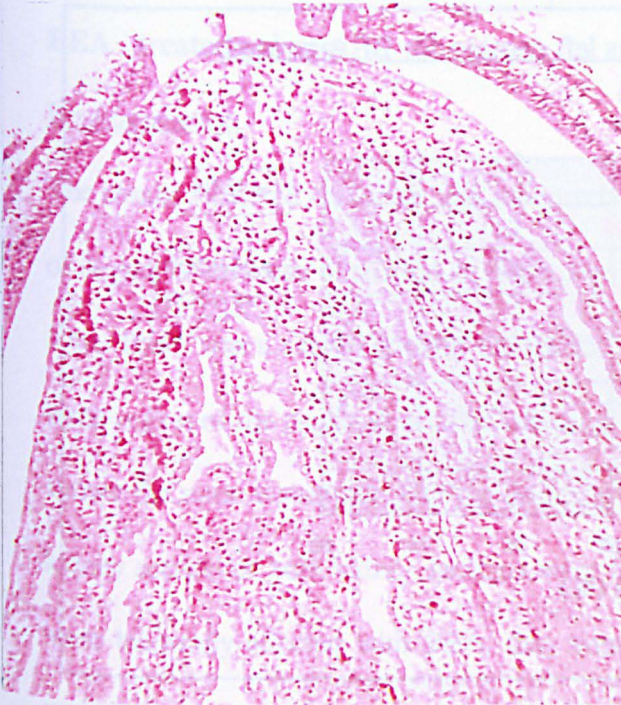


Moderate necrosis: Note loss of interstitial cells; degeneration of some tubules in tip region (arrow), collecting ducts still well preserved. Magnification x 250.

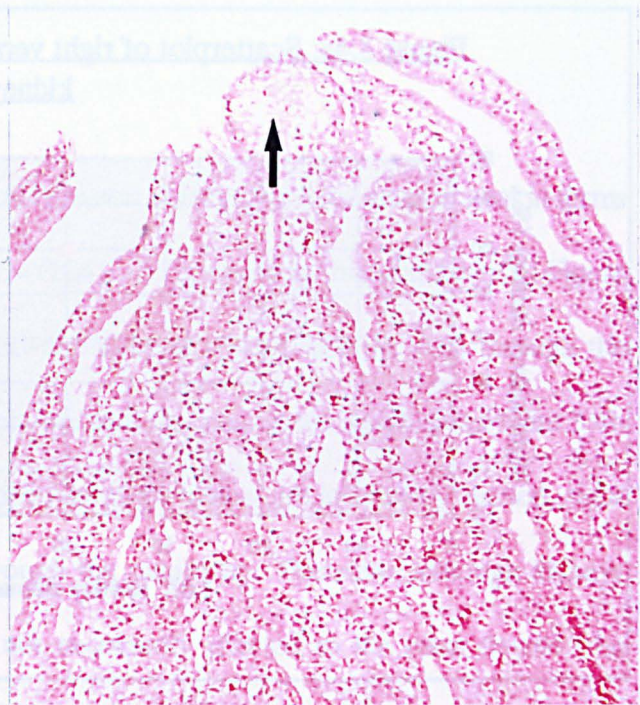


Marked necrosis: Loss of all structural detail in papilla tip area with only ghosts of tubules and blood cells remaining (arrows). Magnification x250.

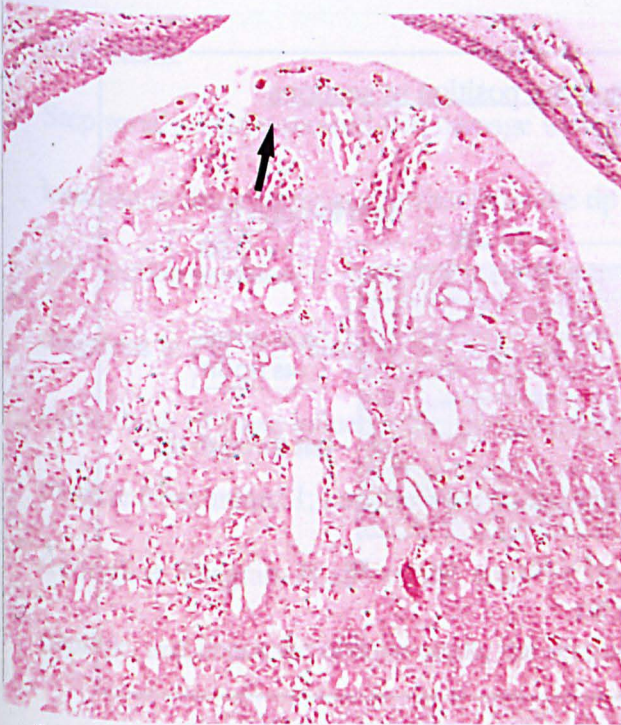
Figure 3.3: Grades of focal papillary necrosis induced with indomethacin



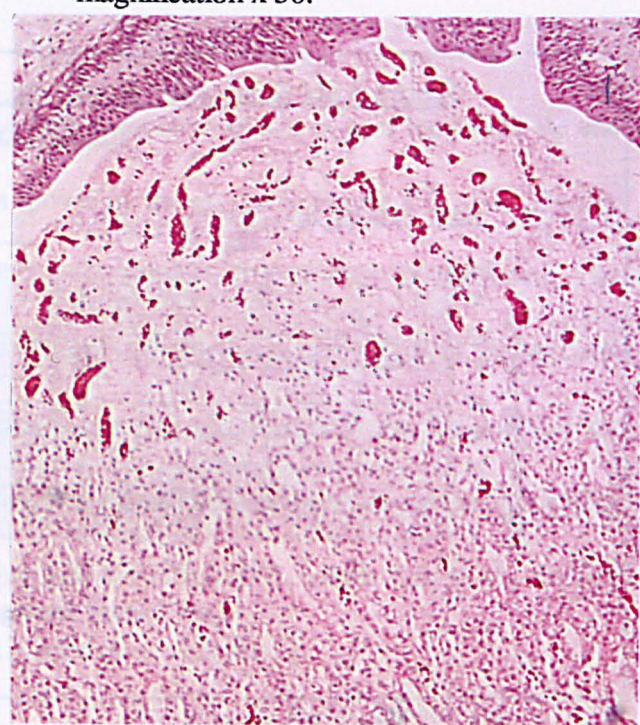
Normal papilla: magnification x 50.



Minimal/mild necrosis: note loss of interstitial detail in some areas (arrow). magnification x 50.



Moderate necrosis: Note loss of interstitial cells and degeneration of tubules in tip region (arrow), collecting ducts still well preserved. magnification x 50.



Marked necrosis: Loss of all structural in papilla tip area with only ghosts of tubules and blood cells remaining. Papilla preserved distal to area of lesion. magnification x50.

Figure 3.4a: Scatterplot of right versus left damage for BEA-treated kidneys

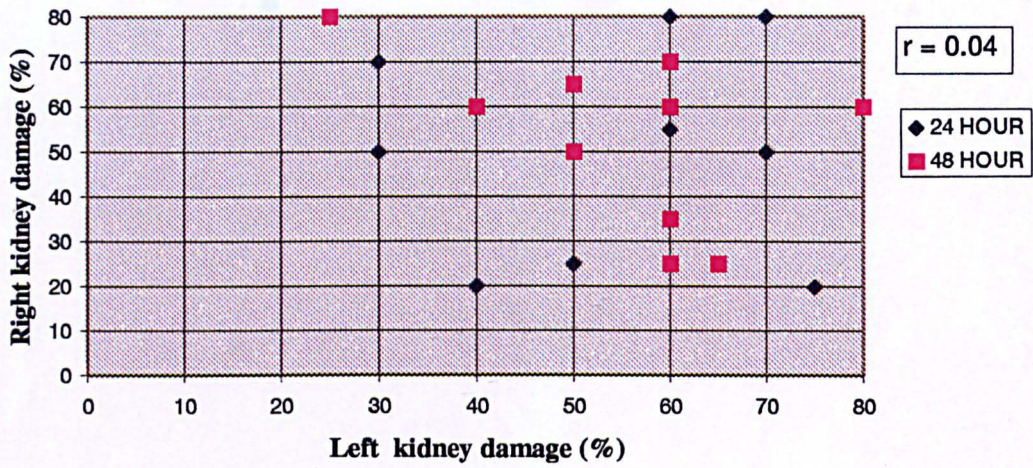
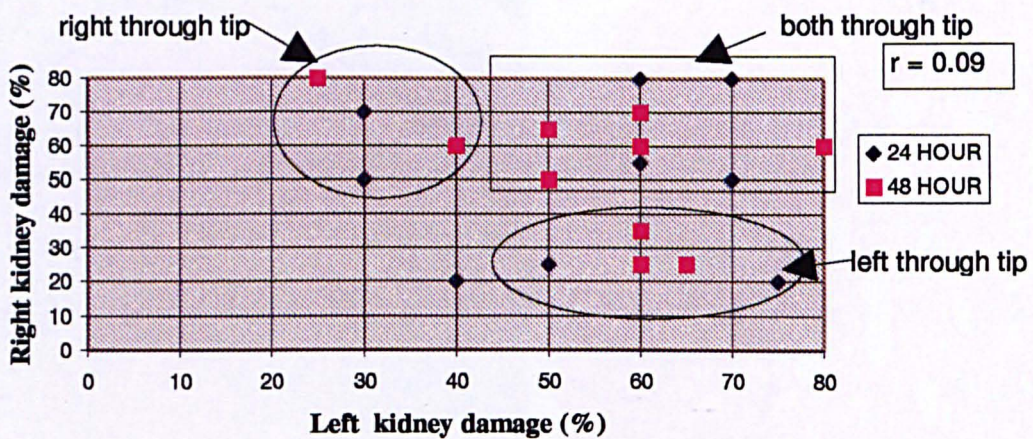


Figure 3.4b: Scatterplot from 3.4a. showing position of sections relative to tip



3.3.1.1; Additional Histology

BEA - treated animals:

Re-examination

The results indicated that there was no correlation between kidneys from the same animal in terms of degree of necrosis, expressed as % damage ($r = 0.04$), as illustrated by the scatterplot in Figure 3.4a. There was a slightly greater correlation between kidneys if both sections were through the same region of the papilla ($r = 0.09$), although this was still not statistically significant; Figure 3.4b shows the same scatterplot with the points grouped to show where the left kidney section, right kidney section or both kidney sections were through the papilla tip. Therefore, although kidneys were bilaterally affected with BEA, they were not necessarily affected to the same degree (the variation within an animal was as great as the variation between animals).

Step serials

Step serial sections showed that damage with BEA was most extensive at or near the papilla tip and lessened as the sections got further from the tip as shown by the graphs in Figure 3.5, plots of score and section position at each 20 micron step-section for each of the 4 kidneys sectioned. The diagrams in Fig. 3.5 illustrate the angle of the papillae in the blocks and the plane of the sections taken; both of these influence the pattern of the plots obtained. Diagram A shows the angle of the papillae from animal 1; papillae were straight and sections started distant from the tip before passing through it. Diagram B shows the angle of the papillae from animal 2, where due to the curved papillae, and hence the oblique angle, the sections started at or near the tip.

The results support the general assumption that BEA damage is most extensive at the tip. It is important to note that this damage gradually lessened further from the tip in a similar manner for all kidneys sectioned (the necrosis score did not markedly alter until sections were well away from the

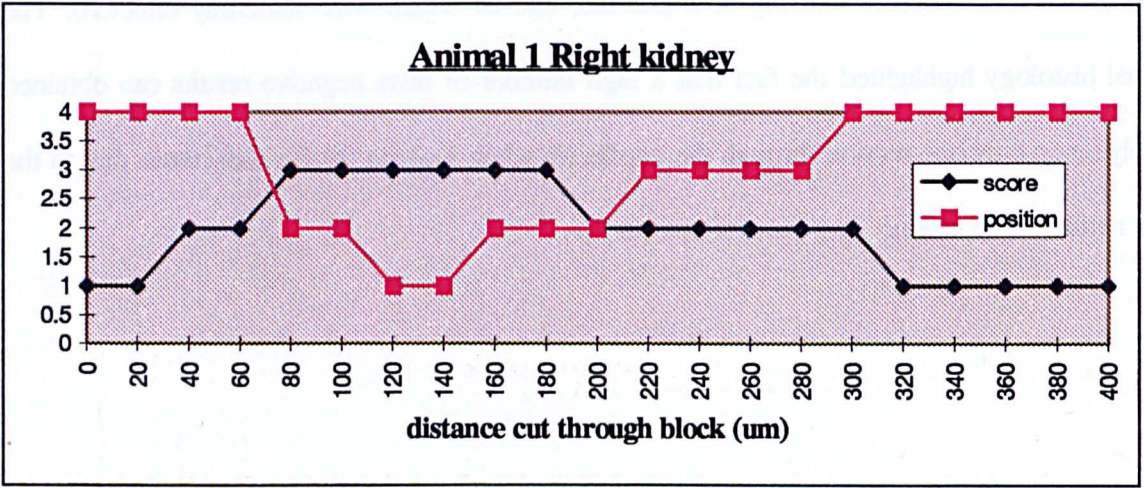
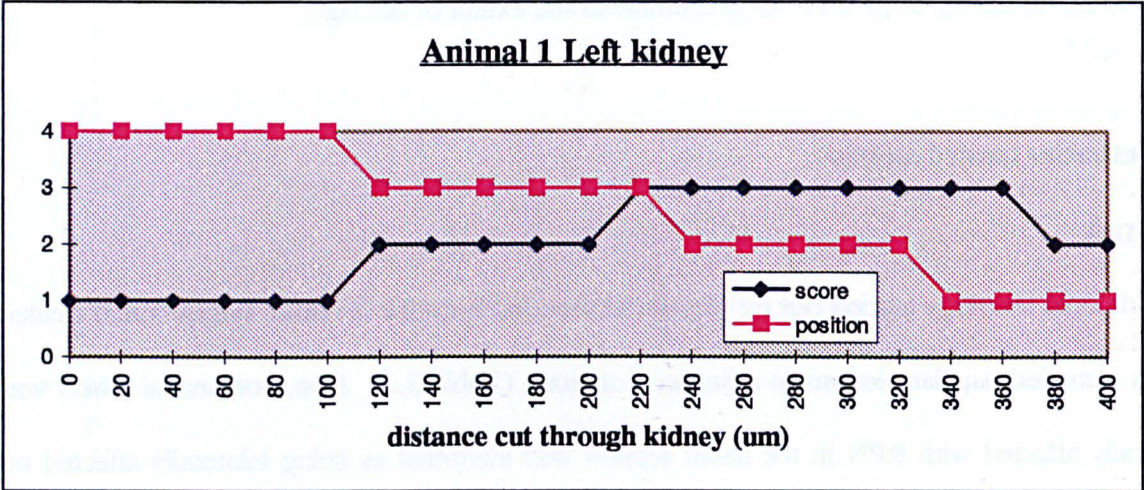
tip). The standard technique used to assess the extent of damage with BEA, a single section near the papilla tip, is therefore robust, since the section does not have to be exactly through the tip, just within 60 μm of the tip, to give a reliable estimate of the extent of damage.

Indomethacin- treated animals:

Step serials

The additional histology carried out on step serial sections from the 24-hour indomethacin-treated animals identified papillary lesions in a further 5 animals (Table 3.5). Also, one animal which was unilaterally affected with RPN in the initial section was identified as being bilaterally affected on further sectioning. The outer cortex was also affected in some animals, but this was not thought to be treatment related (cortical scarring in 3 animals, one of which was bilaterally affected). The additional histology highlighted the fact that a high number of false negative results can be obtained when relying on only one section through the papilla tip when looking for focal necrosis, due to the variable nature of the lesion.

Figure 3.5: Necrosis score and section position for step serial sections through papillae from two BEA-treated animals



Score	Definition	Position	Definition
0	Normal	1	section through tip, showing openings of ducts of Bellini (Area Cribrosa)
1	Minimal necrosis	2	section through tip, but ducts of Bellini not present in section
2	Moderate necrosis	3	section near to tip but not through
3	Marked necrosis	4	section well away from tip

Table 3.5. Indomethacin: additional histology (step serial sections) secondary morphology

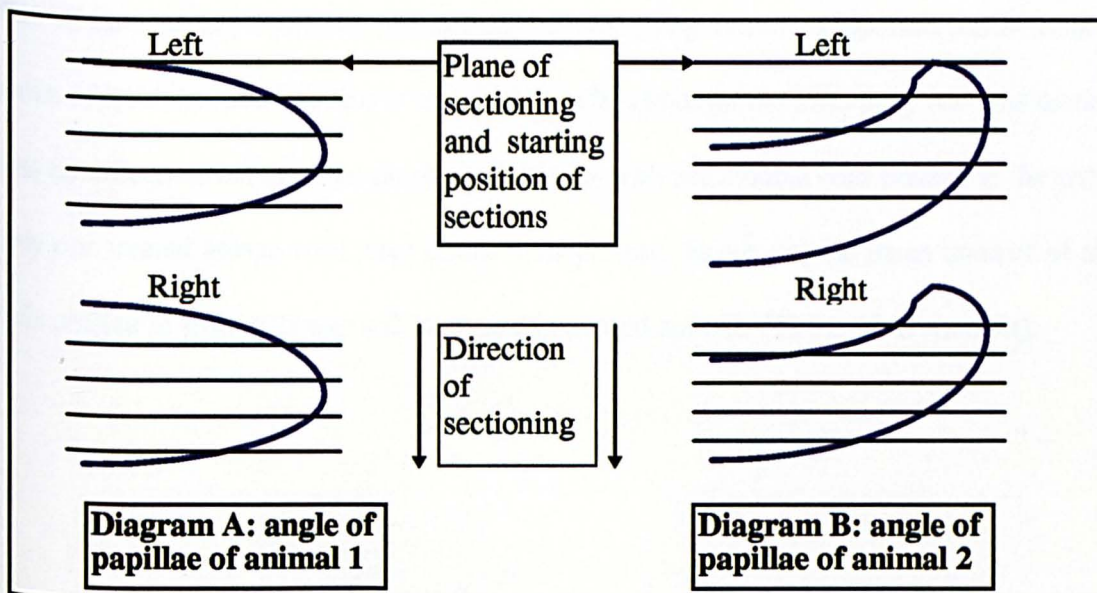
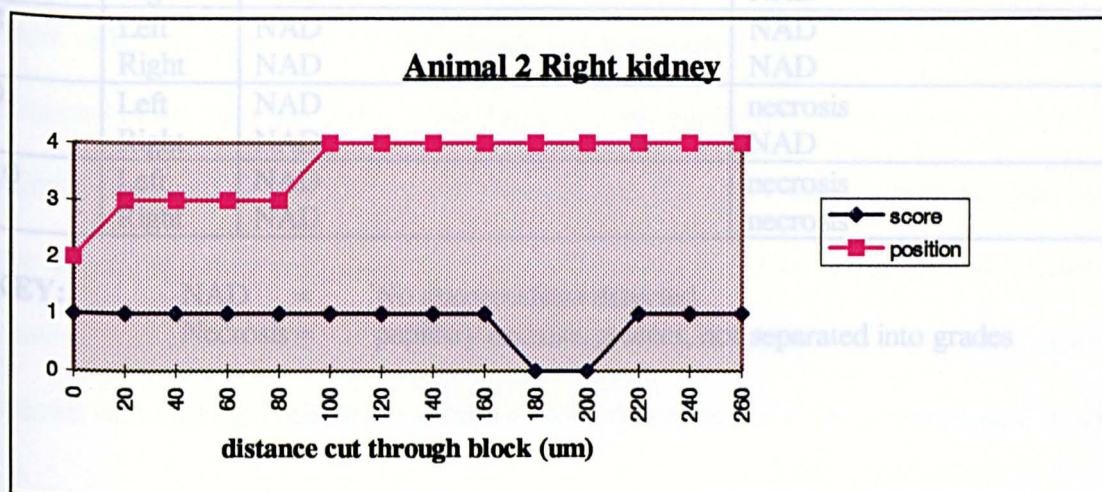
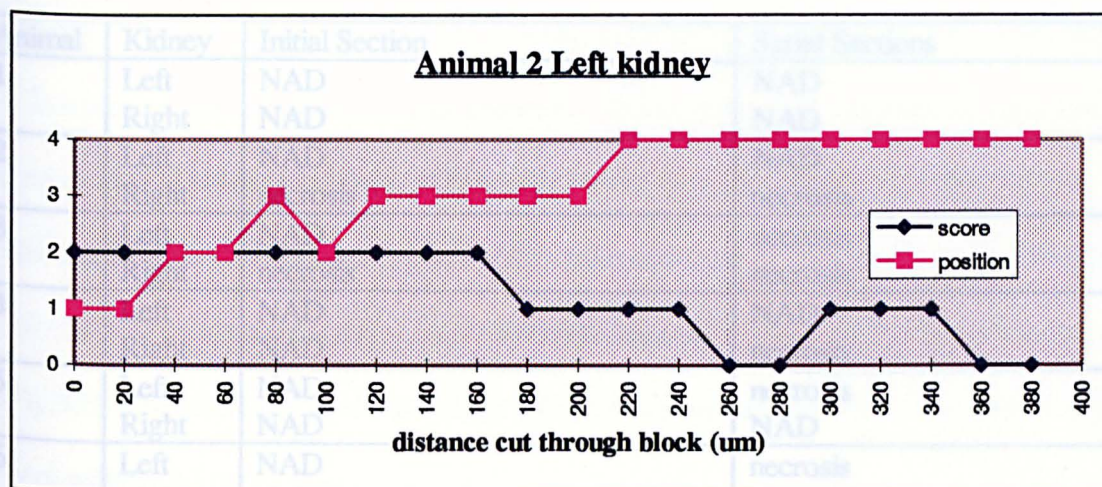


Table 3.5: Indomethacin; additional histology (step-serial sections)- papillary morphology

Animal	Kidney	Initial Section	Serial Sections
1	Left	NAD	NAD
	Right	NAD	NAD
2	Left	NAD	NAD
	Right	necrosis	necrosis
3	Left	NAD	necrosis
	Right	necrosis	necrosis
4	Left	NAD	NAD
	Right	NAD	necrosis
5	Left	NAD	necrosis
	Right	NAD	NAD
6	Left	NAD	necrosis
	Right	NAD	necrosis
7	Left	NAD	NAD
	Right	NAD	NAD
8	Left	NAD	NAD
	Right	NAD	NAD
9	Left	NAD	necrosis
	Right	NAD	NAD
10	Left	NAD	necrosis
	Right	NAD	necrosis

KEY: NAD = No abnormalities detected
 Necrosis = papillary necrosis present, not separated into grades

3.3.2: Urinary sediments

BEA- treated animals:

Urinary sediments from BEA-treated animals collected at 8 hours did not show any obvious differences to control animals, both contained similar types and numbers of cells and only one treated animal had renal tubule cells present in the sediment (Table 3.6 and Fig. 3.6, a plot of individual animals). Sediments prepared from urine collected at 24 hours identified 17 BEA-treated animals with an increase in the number of transitional cells beyond the top of the range for controls (Fig.3.6). The sediments from the 3 other BEA-treated animals contained few cells of any kind and were within the control range.

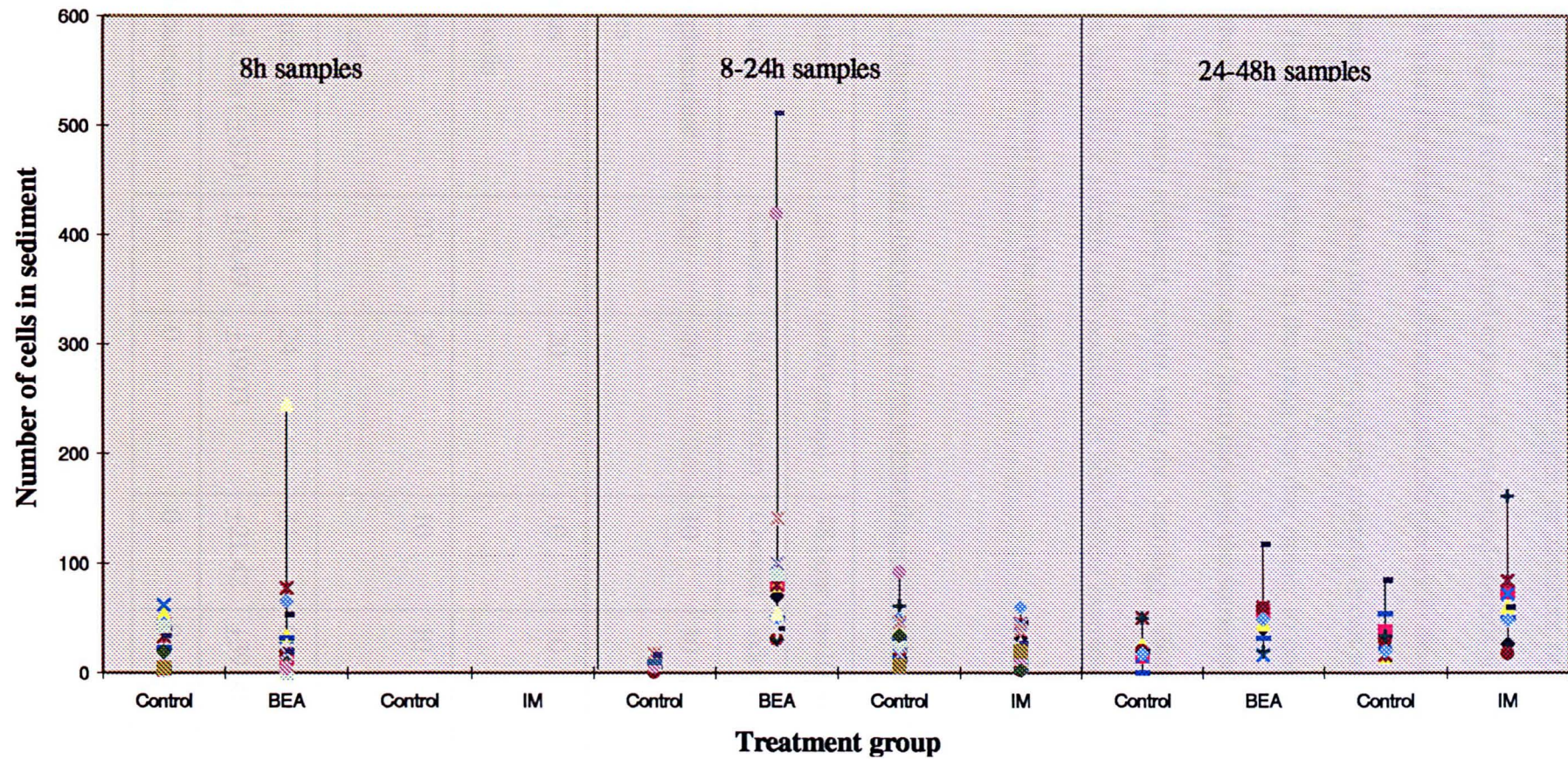
More sediments from BEA-treated animals had transitional cells present than did sediments from controls (19 vs. 14 respectively, Table 3.6) and renal tubule cells were present in 13 of 20 treated animals compared to 0 of 20 controls. Furthermore, the mean number of transitional cells present per animal per group was higher in BEA-treated animals (92 vs. 11 in controls). Correlating the results of the urinary sediments with histology observations, there was a very good relationship; all animals with increased cells in the sediment showed evidence of RPN on histological examination.

The 48 hour urinary sediments taken from the remaining animals supported the 24 hour results in terms of incidence and increase in transitional cells, although the difference was less marked. There was no difference between the number of animals with transitional cells present in the sediment and only one treated animal with renal tubule cells present. However, the mean number of transitional cells present in sediments was still increased in treated animals (49 vs. 25 in controls).

Table 3.6: Urinary sediment observations from BEA-treated animals; incidence table

Observations (presence of)	8 hour (n = 20/group)		24 hour (n = 20/group)		48 hour (n = 10/group)	
	Control	BEA	Control	BEA	Control	BEA
Polymorphonuclear leucocytes	19	19	14	16	10	10
Squamous epithelial cells	20	18	18	20	10	10
Transitional epithelial cells	20	17	14	19	9	10
Mean number of transitional cells in sediment (± SD)	25 ±19.17	40 ±58.4	11 ±6.16	92 ±74.45	25 ±14.25	49 ±28.30
Renal tubule cells	0	1	0	13	0	1

Figure 3.6: Number of cells in urine sediments for each sampling period



Key: IM = indomethacin. Control groups plotted prior to respective treatment groups

Indomethacin-treated animals:

No discernible difference was found in the types or numbers of cells present in the urinary sediments prepared from control and indomethacin-treated animals at 24 hours (Table 3.7 and Fig. 3.6). At 48 hours the mean number of transitional cells present in the sediments from indomethacin-treated animals was higher than those from control animals (mean number of cells = 65 vs. 34 in controls). However, this increase in the mean was due mainly to one animal as can be seen from Fig. 3.6; the range of cell numbers apart from this was the same for treated and control animals. Comparing the urinary sediment results with the histology findings, there was no obvious correlation, since those animals with necrosis on histological examination did not have the highest numbers of transitional cells in the sediment.

Table 3.7: Urinary sediment observations from indomethacin-treated animals; incidence table

Observations (presence of)	24 hour (n=20/group)		48 hour (n=10/group)	
	Control	indomethacin	Control	indomethacin
Polymorphonuclear leucocytes	20	20	10	10
Squamous epithelial cells	20	20	10	10
Transitional epithelial cells	20	20	10	10
Mean number of transitional cells (\pm SD)	31 \pm 19.49	24 \pm 19.03	34 \pm 21.42	65 \pm 39.90
Renal tubule cells	0	0	0	0

Quantification of cell numbers

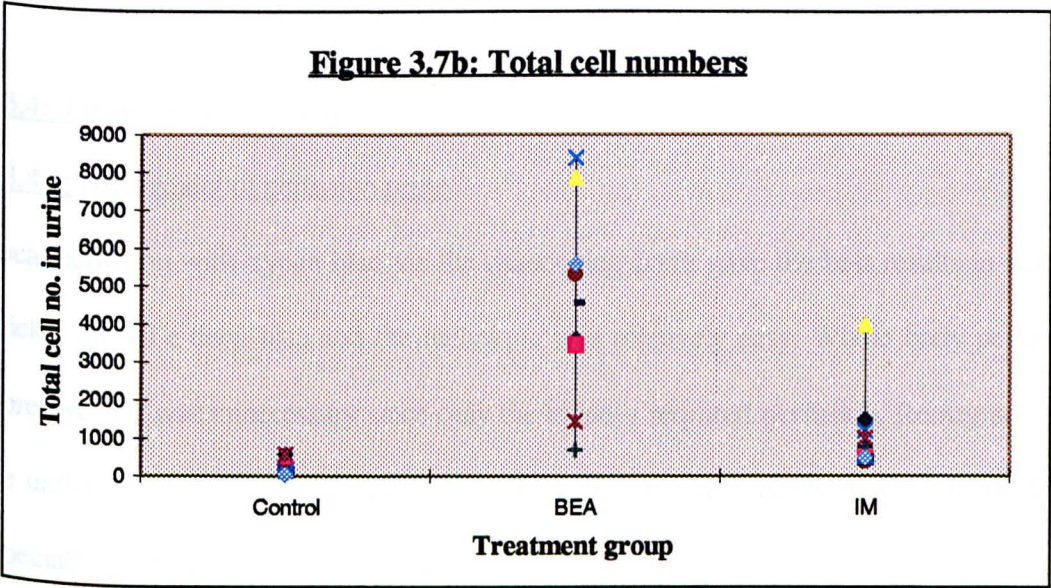
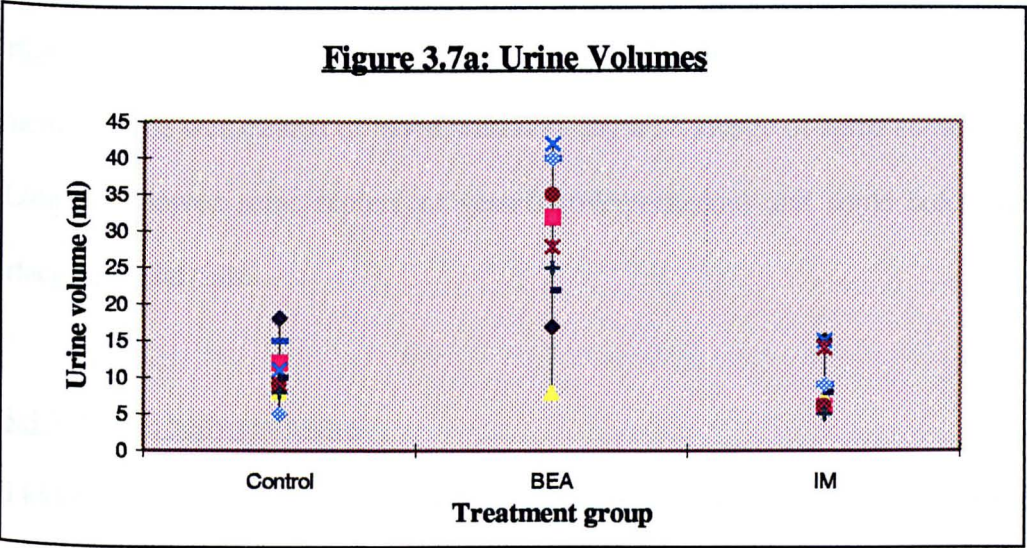
The later urine analysis experiments involving determination of the total number of cells excreted in the urine after 24 hours' treatment gave similar results to those above (Table 3.8 and Fig. 3.7). In BEA-treated animals, the urinary sediments matched well with the histopathology assessments, and the animal with the lowest cell numbers in the urine also had the mildest lesions. The mean volume of urine produced by the BEA-treated animals was significantly greater than the control animal values ($p < 0.001$) with only 2 animals within the control range (Fig 3.7). Indomethacin-treated animals showed no difference in mean urine volume relative to controls ($p > 0.05$). However, there was an increase in the number of transitional cells present in the sediments of indomethacin-treated animals relative to control values due to the values from 4 animals (Fig. 3.7), although this was still not significant. The sediments from BEA-treated animals showed a marked increase in cell numbers over controls with all but 1 animal outside the control range. When the cell number was converted to total number of cells in urine sample, these increases became significant ($p < 0.001$).

Table 3.8: Urinary sediments, quantification of cell numbers

Group (n=10/group, except BEA n=7)	Mean urine volume (ml)	Mean number of transitional cells in sediment (0.25 ml)	Mean total cell number (accounting for volume)
Control	10.50 \pm 3.75	4.80 \pm 4.29	211.60 \pm 197.38
BEA	23.85 \pm 9.23 ***	62.28 \pm 82.93	3854.85 \pm 2406 ***
indomethacin	9.40 \pm 3.86	32.00 \pm 39.44	1070.40 \pm 1089.80

KEY: ***= $p < 0.001$; means given \pm SD

Figure 3.7: Quantification of urine sediments: volume and cell numbers for individual animals



Key: BEA = 2-Bromoethanamine IM = indomethacin

3.3.3: Perfusion experiments

3.3.3.1: Local perfusions

Flow rates of 6 and 10 mL/minute showed good perfusion of the cortex but insufficient perfusion of papilla (some blood cells still remaining), especially at 6 mL/minute. There was also shrinkage of surface epithelial cells and some collecting duct cells. A flow rate of 20 mL/minute showed structures to be well cleared of blood with only slight shrinkage of some surface cells, whereas 25 mL/minute showed some rupture of vessels (overperfusion) and some holes were apparent in surface epithelial cells.

3.3.3.2: Whole body perfusions

All kidneys showed good preservation of the papilla structures with only slight shrinkage of some surface epithelial and collecting duct epithelial cells. Capillaries were well cleared of blood. Both techniques worked well, although whole body perfusion was slightly more straightforward with no need to stop the perfusion to change solutions as was the case with the perfusion pump apparatus.

3.3.4: Trypan blue perfusions

3.3.4.1: Assessment of perfusion route

Local perfusion with trypan blue via the descending aorta gave the best results in that the kidneys quickly turned a deep blue and the technique was relatively easy. Whole body perfusion required more dye and was unnecessary since only the kidneys required perfusion. Retrograde perfusion via the ureter was successful (kidneys turned blue) but was technically more difficult: this would be especially the case in younger rats with smaller ureters.

3.3.4.2: Perfusion experiments

All animals were carefully monitored during anaesthesia to ensure that they maintained a steady,

deep level of anaesthesia and that death had not occurred before perfusion began. All control and indomethacin-treated animals perfused successfully (blood cleared rapidly and kidneys were evenly pale). The papilla of one indomethacin-treated animal showed the presence of a small blue spot of trypan blue when sliced. BEA-treated animals did not perfuse well and only the outer cortex appeared perfused when the kidneys were sliced open.

Histological Assessment:

Kidneys from all control animals were well perfused (with the possible exception of one animal which had some red blood cells present in one papilla). Occasional interstitial cell nuclei in the cortex and cortico-medullary junction (and very occasionally in medulla) were stained with trypan blue. The basement membrane of tubules in the cortex of all animals was also lightly stained with trypan blue (Fig 3.8a).

Kidneys from BEA-treated animals did not perfuse well; the outer cortex only was partially perfused and there was evidence of congested vasculature in the inner cortex and outer papilla (large numbers of blood cells in the medullary ray area) (Fig 3.8b). Some interstitial cells and occasional tubule cells in the outer cortex (diffuse distribution) were stained with trypan blue. All papillae showed evidence of RPN, although this could not be correlated with trypan blue presence due to the lack of perfusion.

Kidneys from indomethacin-treated animals appeared to have perfused well (patent lumen of tubules, no blood cells in cortex), although some red blood cells remained in the papilla, suggesting incomplete perfusion in this area. Some interstitial cells, occasional tubule cells and basement membrane of tubules in the cortex were stained with trypan blue. The papilla of one animal contained 2 structures which were stained with trypan blue, although these were capillaries and not

Figure 3.8: Results of trypan blue perfusions

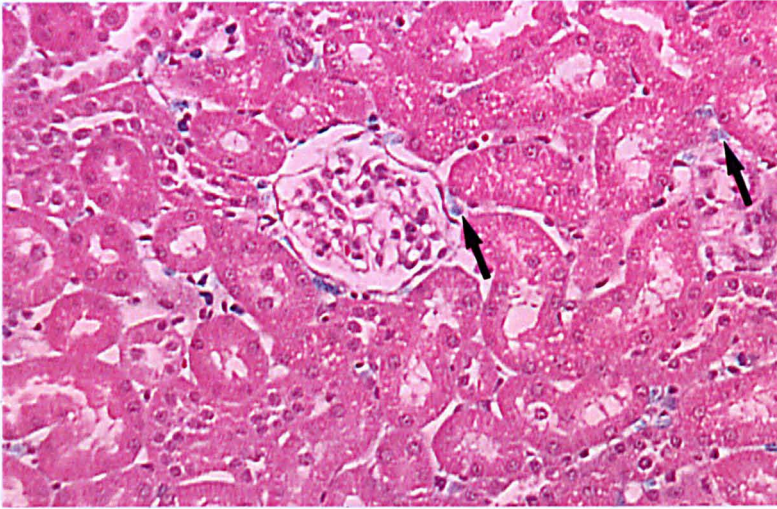


Figure 3.8a: Section from control animal perfused with trypan blue. Note interstitial nuclei stained blue (arrows). H&E, x 50.

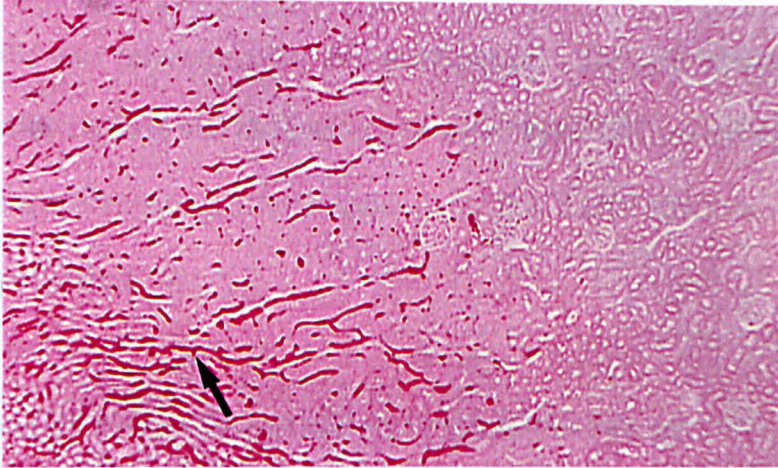


Figure 3.8b: Section from BEA-treated animal perfused with trypan blue. Note lack of perfusion in cortico-medullary area, vessels still congested with blood (arrow). Eosin-stained, x250.

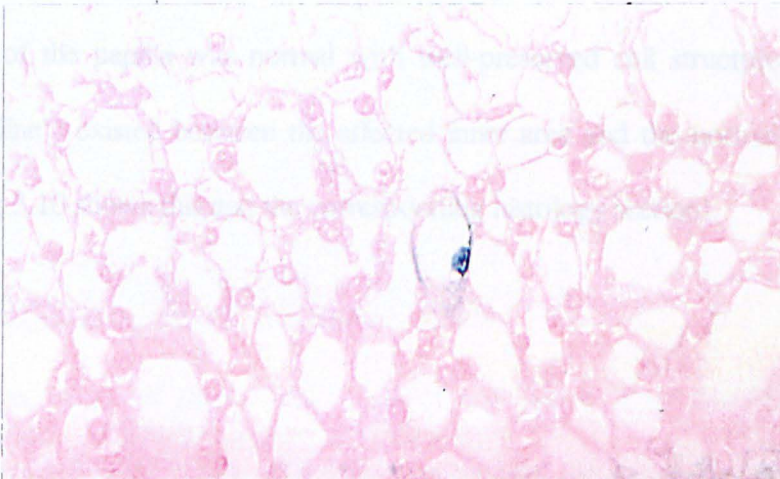


Figure 3.8c: Section from indomethacin-treated animal perfused with trypan blue. Note blue-stained capillary, but no obvious necrosis. Eosin-stained, x 500.

related to any necrotic areas (Fig 3.8c). No other staining was seen in the papillae. No lesions were seen in any of the sections. Trypan blue thus seemed to be of no use in identifying necrosis since it could not be perfused into BEA-treated kidneys and stained areas in indomethacin-treated kidneys that were not necrotic. It was therefore not used in further experiments.

3.3.5: Correlation of scanning electron microscopy and histology

All kidneys processed for SEM showed good preservation of structures with minimal shrinkage of surface epithelial cells of the papilla. Control animal kidneys showed good morphology of all structures, with well preserved and defined tubules and surface epithelium. Kidneys processed through to histology following SEM assessment showed excellent preservation of structures (Fig 3.9 shows SEM and histology from the same sample). The only distinguishing feature from standard histology sections was the presence of a thin black line around the surface of the tissue (the gold coating applied during SEM processing) but this did not affect the assessment. All control kidneys appeared normal.

SEM assessment of the surface epithelium of papillae from all BEA-treated animals showed a varying demarcated effect in that the epithelium of the inner portion of the papillae (nearest the tip) had no obvious cell surface structure (denuded, lost epithelial cells) whereas the epithelium of the outer portion of the papilla was normal with well-preserved cell structure. An obvious border ("constriction line") existed between the affected inner area and the normal outer portion of the epithelium (Fig 3.10 shows this and the corresponding histology section).

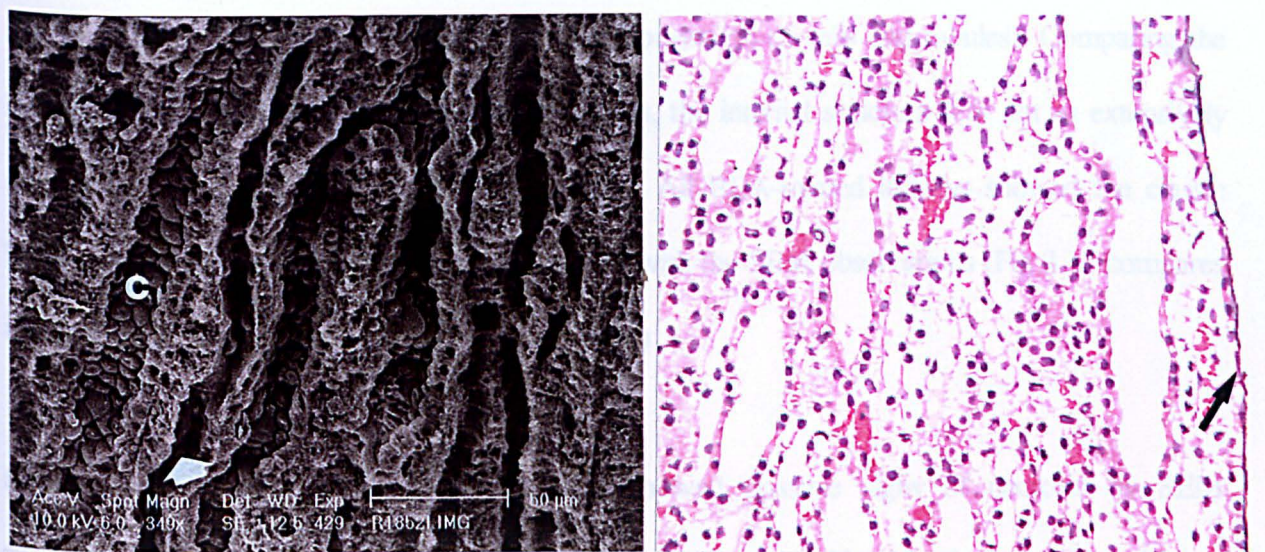


Figure 3.9:

A = SEM photograph from control papilla, showing good preservation of papillary structures (c = collecting ducts, arrow = capillaries)

B = Subsequent H & E section (x 100) from same control papilla, showing good normal structural preservation. Note thin black line at edge of H&E section (arrow), this is the gold coat applied during SEM preparation.

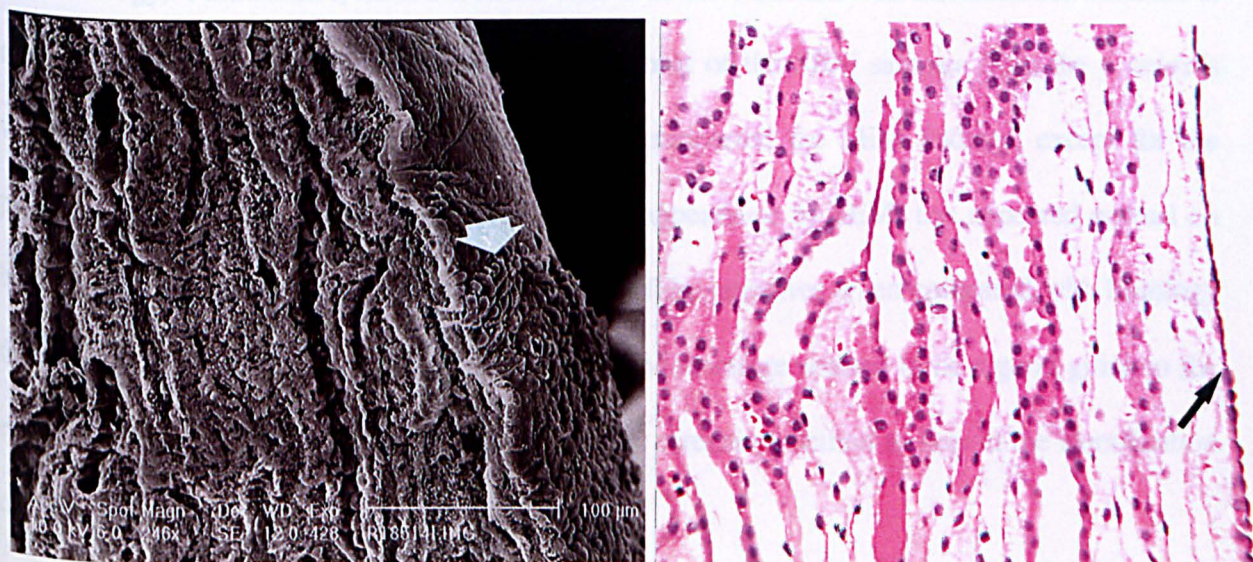


Figure 3.10:

A = SEM photograph of papilla from BEA-treated animal. Note demarcation between normal surface epithelium and sloughed epithelium (arrow).

B = Subsequent H&E section (x 100) from same papilla, showing same border between sloughed and normal epithelium (arrow). Mild RPN is also apparent, note loss of interstitial structures compared to control H&E section in Figure 3.9.

SEM examination of the internal structure of all papillae from BEA-treated animals revealed evidence of RPN, varying from interstitial damage (shrunken cells and congested matrix) to total loss of all tubule structures (with thickening and congestion of cells and tubules). Comparing the internal alterations to the surface epithelial changes, the internal structure was not as extensively damaged as the surface epithelium of the papilla. All BEA-treated kidneys showed the classic histological picture of RPN, and correlated exactly with the SEM observations (Fig 3.11 compares the SEM and histological picture of RPN with BEA).

Kidneys from 3 indomethacin-treated animals showed possible signs of necrosis by SEM examination (loss of interstitial and some tubule structures) with alterations or distortion of interstitial structures in a further 6 animals. A small area of papillae of one of these animals showed only slight interstitial alteration ("foamy" appearance). The 3 indomethacin-treated kidneys which had shown signs of necrosis with SEM were confirmed as having RPN or marked alteration (interstitial degeneration) in the histological sections of the same samples (Fig 3.12 compares SEM and histology). Furthermore, these 3 animals were 3 of the 4 animals with increased cell numbers in the urine, as reported in 3.3.3. Histological assessment of the SEM samples from the 6 animals which had shown possible alterations in structure supported the SEM findings, except for the papilla which had been noted as having a "foamy" appearance by SEM; this appeared normal on histological examination. SEM did not produce any false negatives when compared with histology from the same samples, even with small lesions. SEM therefore seemed to be a good guide to the extent of damage within the papilla and was an easy screening method to help in the identification of necrotic areas.

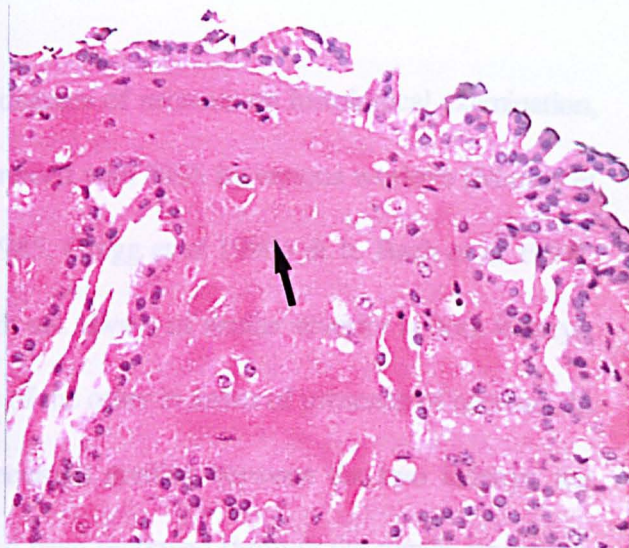
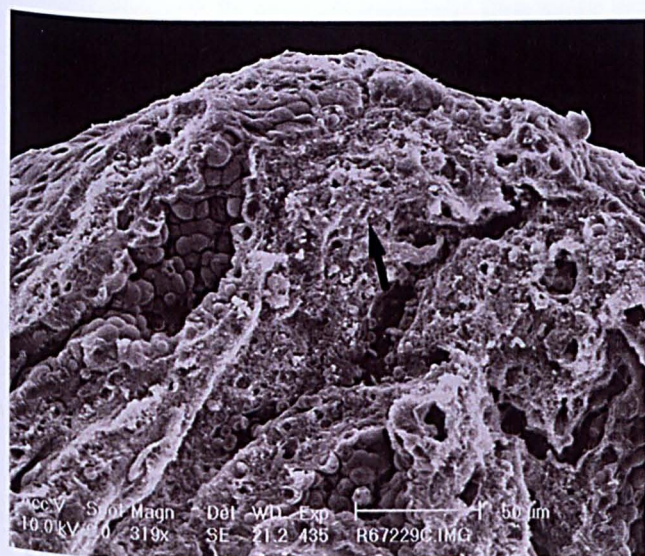


Figure 3.11:

A = SEM photograph of papilla from BEA-treated animal: **B** = Subsequent H & E section (x 250) from same papilla, showing moderate RPN (arrow). Note the congested, disorganised appearance of interstitium and tubules in area behind tip in SEM photograph (arrow): this corresponds to the area of moderate RPN shown in the H&E section from the same sample.

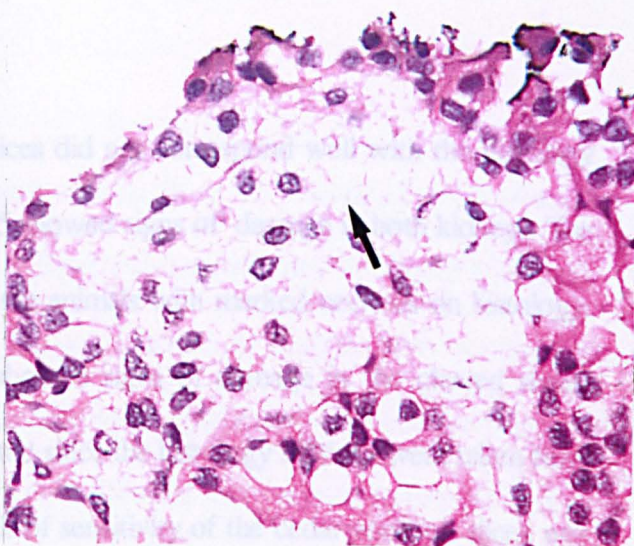
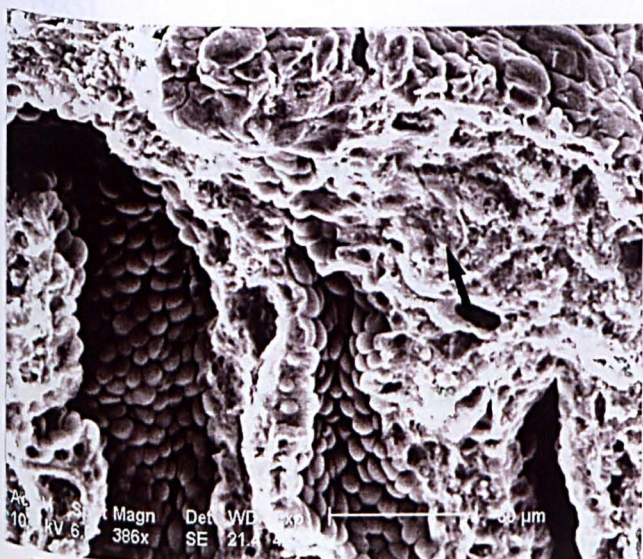


Figure 3.12:

A = SEM photograph of papilla from indomethacin-treated animal: **B** = Subsequent H&E section (x 500) from same papilla, showing mild, focal RPN (arrow). Note disorganised appearance of area of interstitium immediately behind tip in SEM photograph (arrow); this corresponds to the area shown in the H&E section.

3.4 DISCUSSION

3.4.1: Histology

BEA-treated animals

All BEA-treated animals showed signs of varying degrees of necrosis on histological examination, comparable to that previously reported in rodents treated with BEA by various workers [Murray et al 1972, Mattingley et al 1984, Bach & Bridges 1985, Gregg et al 1990a & b, Bach et al 1991]. Necrosis was bilateral in all treated animals although not always of similar severity in both kidneys, suggesting that one kidney is a reasonable predictor of the other in terms of presence of necrosis. In the animals where there was a difference in the necrosis grading between the two kidneys, the kidney with the most severe grading ("marked") had been sectioned through the papilla tip, which is the area most sensitive to necrosis [Duggin 1980]. Further sectioning of the kidneys graded as "mild" revealed that necrosis became moderate or marked when sectioned through the papilla tip, and therefore of similar severity to the contralateral kidney. This reinforces the importance of standardising sectioning for assessment of necrosis, as discussed by Bach & Bridges [1982 & 1985].

Findings from the cedarwood oil-cleared kidney slices did not correspond well with the histology findings. Of the 20 animals treated with BEA, only 5 showed signs of damage in both kidneys. The cedarwood oil-cleared slices were more reliable in the animals with marked necrosis on histology: 10 out of the 12 kidneys with marked necrosis showed signs of damage in the cleared slices. However, better correlation might have been predicted since the histology sections were taken from the cedarwood oil slices. One problem with the lack of sensitivity of the cedarwood oil slices was the thickness: in order that sufficient tissue was available to process and cut histology sections, the kidney slices had to be more than 1mm thick. At this thickness, it was difficult to discern much of the kidney structure, even using Nomarski microscopy.

Re-examination of BEA-treated kidneys to determine how close the two kidneys from each animal were in terms of damage indicated that there was no correlation between them ($r = 0.04$). Thus, although kidneys were always bilaterally affected with BEA treatment, they were not necessarily affected to the same degree (the variation within an animal was as great as the variation between animals). Step-serial sectioning, carried out to determine how much the distance from the papilla tip affected the extent of necrosis, showed that damage was most extensive in the papilla tip region. This supports the general assumption that papillary necrosis induced with BEA is most extensive at the tip [Hill et al 1972, Duggin 1980, Bach & Bridges 1982 & 1984]. It is also important to note that this damage became gradually less as the lateral distance from the tip increased in a similar manner for all the kidneys sectioned, since the damage score did not markedly alter until the sections were well away from the tip. The standard technique used to assess the extent of damage, a histological section near the papilla tip, is therefore robust since the section does not have to be exactly through the tip, just in the tip region (within 60 μm of the tip), to give a reliable estimate of the extent of damage.

Indomethacin-treated animals

The histology findings from the initial and additional sections from the kidneys of indomethacin-treated animals highlight the relatively high number of false negatives obtained histomorphologically when relying on one section through the tip with this focal model, as discussed by Arnold et al [1974]. Initial sections from the 24 hour time point indicated 2 animals were affected, yet the extra sections identified a further five. Such serial sectioning studies are very time consuming (1 day to cut and assess 1 kidney block) and not really feasible on a large scale. However, the information obtained is important in highlighting both the limitations of relying on a single section for studies involving focal necrosis and reinforces the need for more reliable techniques in identifying such

damage [Arnold et al 1974, Bach & Bridges 1982 & 1985, Price et al 1996].

3.4.2: Urinary sediments

Urinary sediments from BEA-treated animals gave a reasonable correlation with the histological findings and proved useful in indicating those animals where necrosis had, or was, occurring. Urinary sediments taken at 24 hours proved most useful in identifying possible renal papillary necrosis; 17 out of the 20 BEA-treated animals had some signs of necrosis; the sediments from the 3 animals where necrosis was not evident were very scant, with few cells of any kind present, so these may not have been representative samples (or there may actually not have been any cells in the urine). At 48 hours the differences between BEA-treated animals and controls were less marked, although there was still an increase in the mean number of transitional cells present in sediments (49 vs. 25 in controls).

The numbers of transitional and renal tubule cells present in the sediment from each animal generally gave some indication of the extent of damage present in the histology sections; those animals with the highest numbers of transitional cells showed either moderate or marked necrosis on examination of the histology sections. This supports work which looked at the significance of urinary cell excretion with BEA and other renal toxins and found that there was a broad agreement between the numbers and types of cells excreted and the severity of damage [Davies et al 1968, Kennedy & Gail Saliya 1970]. The results from the urinary sediments suggest that most damage (measured by amount and type of cells passed out in the urine) occurs 8-24 hours after treatment with BEA. This correlates well with the work of Davies et al [1968] and Kennedy & Gail Saliya [1970], who also suggested that most of the damage following BEA treatment occurred within the first 2 days of treatment.

The initial results from examination of the urinary sediments from indomethacin-treated animals suggested that this technique was of little value in identifying focal papillary necrosis since the numbers and types of cells present in the sediment were not obviously increased in treated animals. This is perhaps due to the nature of the lesions induced with indomethacin in terms of size and frequency. However, urinary sediments gave promising results with both BEA and indomethacin when the total number of transitional cells excreted was calculated. All urine samples from BEA-treated animals contained high numbers of transitional epithelial cells (including renal tubule cells) and all kidneys had extensive RPN, coupled with a marked increase in urine volume (polyuria). Polyuria is a well-recorded feature of BEA-induced nephrotoxicity [Sabatini 1984, Bach & Bridges 1985, Wilks et al 1984 & 1986, Stonard et al 1987] and has been reported as the earliest effect of BEA, occurring within 3 hours of treatment [Wilks et al 1986]. Four indomethacin-treated animals had higher numbers of transitional cells relative to controls when corrected for total urine volume, and kidneys from 3 of these animals had some kind of abnormality or necrosis present.

Urinary sediments have been used in humans and in animal studies to investigate and help indicate the presence of various types of kidney damage, but have often been compared with urinary enzyme levels rather than with histopathology findings, as discussed in the introduction to this chapter. Of direct relevance are the findings of Jackson et al [1978], who studied urine cytology in analgesic nephropathy with regard to carcinoma screening and recommended its use as a screen for possible carcinoma or dysplasia in analgesic abusers with RPN (i.e. those patients at risk of developing urothelial malignancies). They used Papanicolaou-stained preparations (as in the present experiments) and found a trend; more severe cytology changes were found in cases of more severe renal failure. They suggested that urinary sediments might therefore also be useful to help detect RPN in patients, a suggestion that is supported by the results of the present experiments with BEA and indomethacin.

Fent et al [1988] and Zbinden et al [1988] assessed the use of "quantitative" assessment of the urine (volume, pH, enzymes, proteins, cells present) and compared the results with histology data. They found that cell excretion and presence of cylinders were the most sensitive markers for detecting nephrotoxicity, but emphasised that cell excretion and enzymuria can fluctuate or disappear with continued treatment. The findings of Davies et al [1968] and Kennedy & Gail Saliya [1970] and those of the present experiments are a good example of this transient cell excretion, showing that the biggest increases in urinary cell excretion with BEA occurred within the first 1-2 days of treatment and then reduced to control levels. Transient enzymuria was also reported following an initial dose of the cortical toxins para-aminophenol and mercuric chloride [Cottrell et al 1976], and urinary enzyme measurements were suggested to be of limited value because of this. Stonard et al [1987] have recommended the daily collection of urine samples to enable the onset of, and recovery from, renal lesions to be followed and to identify critical sampling times so that such transient effects are not overlooked.

Fent and Zbinden and co-workers [1988] emphasised that histopathological examination of the kidneys was an important source of information regarding the nature, localisation and reversibility of lesions, but was a less sensitive screening tool than urine analysis. They commented that since urine analysis was performed during the course of their study and histopathology was performed at the end, a structural correlation with the urine findings was not possible. However, the present experiments with BEA and indomethacin involved urine analysis and histopathology over the same time periods, which made comparison of histopathology and urine findings possible; urine sediments generally correlated well with the histology findings.

Fent and Zbinden & co-workers [1988] also highlighted the problems of using "unit volume" (cells per aliquot of urine) when expressing cell excretion; total urine volume must be measured and an

estimate obtained of total cells excreted to get an accurate measure of any effects. The results of the present experiment emphasised the importance of this. When cell numbers were corrected for urine volume (total cells excreted) the increase in cell numbers in the urine of BEA-treated animals became more significant, and sediments from indomethacin-treated animals showed an increase in total cell numbers which was not apparent when expressing cell number relative to unit volume.

3.4.3: Perfusion experiments

At lower perfusion rates using perfusion pump apparatus, there were indications that the perfusion pressure was not ideal for the papilla: the histology sections showed that some blood cells remained in the papilla, and the SEM preparations showed collapse of some structures, suggesting insufficient perfusion pressure. It has been demonstrated that higher pressure is needed for perfusion of the papilla compared to cortex, and that different regions of papilla require solutions of different osmolality [Maunsbach 1966, Bohman 1974], although the effects of different perfusion pressures were not investigated. It may therefore not be possible to obtain optimal perfusion of all structures within the papilla.

The results of the perfusion experiments demonstrated that immersion fixation is preferable to using perfusion fixation if the latter is at insufficient pressures, and provides acceptable results with SEM examination. If it is impossible to optimally perfuse the whole papilla, especially when looking at the whole structure as is the case with SEM, it may be that immersion fixed material is preferable. This means that archive material which has been immerse-fixed is perfectly suitable for SEM assessment, and that this technique can be used in routine studies without the need for specialised fixation techniques.

Comparing the methods of perfusion (perfusion pump and fixed head apparatus), local perfusion is

recommended where kidneys need to be treated differently to allow greater control, e.g. one perfused with dye and one not, or if only kidneys are required to be perfused and other tissues must be unfixed e.g. for biochemical analysis. Whole body perfusion is recommended for rapid perfusion with minimal intervention necessary once perfusion has begun: this would be particularly suitable for transmission electron microscopy investigations.

3.4.4: Trypan blue perfusions

Perfusion with trypan blue was of little success, partly due to the problems with perfusion in the BEA-treated animals. This is in contrast to the success reported with trypan blue perfusion in identifying necrotic cells in the liver [Belinsky et al 1984].

It was interesting (and perhaps not surprising as discussed below) to find that the lack of perfusion of the papillae of BEA-treated animals appeared to be due to a vascular effect in the inner cortex area, thus preventing perfusion of vessels in the papilla. This is a useful observation in terms of effects of BEA on the vasculature and subsequent development of lesions even if it means that perfusion cannot be used for such animals (back-perfusion via the ureter is an alternative but this was a difficult and unpredictable means of perfusion). The observed vascular effects could be due to a number of conditions: vasoconstriction; congestion; thrombosis or some other blockage of the vasculature. The effects of BEA on the vasculature of the kidney have been investigated previously in attempts to identify the possible causes of RPN induced with BEA and extrapolate this to RPN in humans, often with conflicting results.

Cuttino et al [1981] used microangiography and tubular micropuncture to investigate whether the cause of RPN was due to vasoconstriction of medullary vessels with ischaemic necrosis. Decreased perfusion of the vasa recta, with unobstructed tubules, was seen during the early stages of RPN,

with vascular obliteration, intratubular debris and tubular obstruction in the late stages. Cuttino et al [1981] therefore suggested that RPN with this model is caused by vasoconstriction rather than direct tubular toxicity. Russell et al [1987] reported that BEA induced an increase in vascular resistance and decrease in blood flow in the kidneys, leading to an increase in blood pressure. However, Sabatini [1984] found that medullary blood flow was not altered by BEA treatment, despite apparent engorgement of vessels.

Wyllie et al [1972] investigated the effects of reserpine (a catecholamine depletor which prevents vasoconstriction) on BEA-induced RPN and found it had a pronounced inhibitory effect on the development of the lesion. They suggested that vasoconstriction may therefore play a significant role in the development of RPN after BEA treatment, but stated that the evidence was incomplete. This work was supported by Wolf et al [1991b & 1992a] who also investigated the influence of reserpine. Wolf and co-workers [1991b & 1992a] suggested that vasoconstriction contributed to the development of BEA-induced cortical tubular necrosis in mice [1991b], and proposed ischaemic necrosis as the cause of BEA-induced RPN in gerbils [1992a]. However, Solez et al [1974] reported that although reserpine inhibited the development of RPN, it had little effect on medullary plasma flow. They stated that their observations did not support the hypothesis that vasoconstriction is the mechanism for BEA-induced RPN, at least in the early stages of damage. They proposed the mostly likely mechanism to be concentration of BEA in the papilla, where it has a direct toxic effect. They suggested that reserpine decreased the incidence of RPN because of stress or, if BEA has a direct toxic effect on cells, it could prevent the uptake and storage of BEA. These conflicting arguments regarding the role of vasoconstriction emphasise the problems in establishing the mechanisms of RPN, as discussed in the introductory chapter of this thesis.

It was hoped that perfusion of kidneys from BEA-treated rats with trypan blue would give a good

indication of the usefulness of this technique in identifying extensive RPN, but since the papillae did not perfuse this could not be verified. There were no such perfusion problems with the indomethacin-treated animals. The histology sections from the indomethacin-treated animals did not contain any areas of necrosis. However, due to the focal nature of the lesions produced with this compound, step serial sections are usually necessary to get an accurate picture of the degree of damage (as highlighted by Wilks et al [1986] and the additional histology carried out on indomethacin-treated kidneys in the present experiments).

The papilla of one indomethacin-treated animal showed two structures stained with trypan blue, but these appeared unrelated to any necrosis. Step serials through the remainder of this block may have eventually shown some necrosis in this area, however the aim of using trypan blue was to save time by highlighting areas of necrosis, not to necessitate time-consuming extra work to determine its usefulness. The lack of trypan blue staining may have been due to leaching out of the dye into the fixative on storage, or to too weak a concentration of dye. However, trypan blue staining was observed in some interstitial nuclei in the cortex of kidneys from control animals, where there was no evidence of necrosis. Therefore although trypan blue had been successfully perfused into the kidneys, it was retained in some healthy cells rather than excluded by them. The results of these experiments indicate that trypan blue is too unreliable for use in routine studies, and it is therefore recommended that trypan blue not be investigated further for use in the identification of necrotic renal lesions.

3.4.5: Correlation of SEM and histology

Scanning electron microscopy (SEM) gave very promising results with both treatment groups, particularly when combined with secondary histology (reprocessing of SEM tissue for histology). All kidneys from BEA-treated animals were identified as having RPN by SEM and secondary

histology of the samples confirmed this. All gradings of RPN were generally in agreement. SEM examination of BEA-treated animals highlighted the sloughing effect on the surface epithelium and its relationship with the effects on the internal papilla structures. This effect of BEA on the surface epithelium correlated well with the histological sections which showed loss of epithelium from the papilla. Loss of surface epithelial cells, following initial hypertrophy, is a well documented effect of BEA [Bach & Bridges 1982 & 1985]. The observation that the surface changes occur where the tubule structure is normal suggests that the surface effects may precede and possibly be a contributory factor in the effects on the tubules and interstitium, by rendering the underlying papillary structures more susceptible to damage.

SEM of the indomethacin-treated kidneys proved very useful in identifying abnormal areas within the papillae. Not all of these were necrotic, but subtle changes in structure, such as gaps in some interstitial areas, were detectable. All of the changes identified by SEM were confirmed by secondary histology. Some of these kidneys were classed as "NAD" by primary histology, but this assessment was of only one half of the papilla (the other half was processed for the SEM/secondary histology). It is possible that the changes seen were only present in the part of the papilla used for SEM and secondary histology due to the focal nature of indomethacin damage. Perhaps the most important observation is that SEM did not produce any false negatives, even with very small lesions. It is possible that SEM is of more use since a much thicker portion of papilla is viewed, giving a "three-dimensional" picture of the necrotic area, thus providing the same amount of information as several step serial histology sections through the papilla.

SEM has been used by others, with successful results, to evaluate the changes in surface epithelial cells of the renal pelvis following treatment with proliferating agents [Shibata et al 1989] and to look at bladder epithelial changes following acute toxicity with tetraethylorthosilicate [Okamura et

al 1992]. It has also been used to examine infection of the papilla associated with RPN [Cohen et al 1979 & 1981]. However, no references could be found for the use of SEM to investigate RPN per se, nor for the combination of SEM and correlative histological assessment.

SEM therefore seems to be an effective and potentially useful technique for identifying and investigating lesions in the papilla, particularly when combined with correlative light microscopy of the same tissue following SEM assessment. The results obtained from the correlative histology performed confirmed that areas identified as "abnormal" by SEM were seen to be affected in some way using histology. Perhaps more importantly, the technique of correlative SEM/histology from the same tissue has potential for other studies.

3.4.6: Techniques recommended for further investigation

The results from both BEA and indomethacin experiments suggest that assessment of urinary sediments and SEM are both useful techniques which can aid in the identification of renal papillary necrosis in both extensive and focal RPN. Perhaps more importantly, SEM can be combined with secondary histology, (which is of equal quality to standard histology) so that no information is lost by performing this technique (SEM and histology are normally performed as mutually exclusive techniques). SEM can be performed on standard study material (i.e. tissue immerse-fixed in formalin) with no loss of detail, which makes this technique applicable to any routine studies where RPN or any other type of necrosis may be suspected.

The next stage in evaluating these techniques was therefore to use them in conjunction with ethoxyquin, a papillotxin which has not previously been investigated in detail as a model for RPN, and compare the results with those obtained in this chapter using the established models BEA and indomethacin.

CHAPTER 4. ETHOXYQUIN STUDIES

4.1 INTRODUCTION

4.1.1: Ethoxyquin

Ethoxyquin, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, is a synthetic anti-oxidant which is commonly used for preserving animal feeds because of its ability to prevent oxidative degradation of vitamins and polyunsaturated fats [Skaare 1976, McIntosh et al 1986]. It is normally added to feed up to a maximum concentration of 150mg/kg feed (0.015%), which is considered a safe level [McIntosh et al 1986]. Its toxicity, metabolism and potential pharmaceutical uses have been investigated in some detail over the last 30 years. More recent investigations have shown ethoxyquin to be capable of inducing RPN [Hard & Neal 1990 & 1992, Manson et al 1992] but its potential as a model for RPN, the development of RPN with ethoxyquin, and the early changes associated with this have not been investigated in detail.

4.1.2: Metabolism and reported toxicity of ethoxyquin

The first reported toxicological assessments of ethoxyquin were conducted by Wilson & DeEds [1959] and Wilson et al [1959] who looked at the toxicity of ethoxyquin and its absorption, metabolism and excretion. For the toxicity assessment [Wilson & DeEds 1959], ethoxyquin was given to rats by oral gavage at doses ranging from 125 to 1000 mg/kg. They reported deaths at 1000 mg/kg (5 of 7 rats), 800 mg/kg (2 of 5) and 640 mg/kg (1 of 5). The clinical signs reported were loose stools, and "depression" at Day 2 of treatment. Dark urine was also reported in rats following ethoxyquin treatment, which was said to be due to a metabolite of ethoxyquin but was not discussed further [Wilson et al 1959]. The toxicity of ethoxyquin in mice, using i.p. dosing, was also investigated, with 5 of 7 deaths reported at 1000 mg/kg but no deaths at 800mg/kg or below. They therefore proposed that rats were

more sensitive to the toxic effects of ethoxyquin. Rats have since been used almost exclusively in subsequently reported work with ethoxyquin.

The metabolism work conducted by Wilson et al [1959] was carried out in rat and cow. They reported that ethoxyquin was rapidly and almost completely excreted in the urine and faeces, with little breakdown to CO_2 , suggesting a stable ring structure. Its distribution in tissues suggested breakdown to a more soluble molecule (the metabolite mentioned above as the possible cause of brown urine), but this was not expanded upon. Ethoxyquin was reported to undergo rapid metabolism and modification, with different spots demonstrable by chromatography in 24 hour urine samples. The turnover time of a single dose of ethoxyquin was reported as 10 days; continuous dosing for 10 days gave increased ethoxyquin concentrations in liver and kidney, suggesting ethoxyquin metabolism occurs here.

Further metabolism studies [Lin & Olcott 1975], which supported those of Wilson and co-workers [1959], reported that ethoxyquin was easily oxidised to a very stable free radical, ethoxyquin nitroxide (EQN), by the substitution of N-O^\bullet for N-H in its structure. This could therefore be the metabolite mentioned by Wilson & co-workers [Wilson & DeEds 1959, Wilson et al 1959]. Lin & Olcott postulated that the mechanism of action of ethoxyquin involved its free radical, EQN, which is a much more potent antioxidant than ethoxyquin itself.

The distribution of ^{14}C -ethoxyquin in adult male rats was investigated using whole body autoradiography and liquid scintillation counting [Skaare & Nafstaad 1979]. Ethoxyquin was given by oral gavage in arachis oil at a dose of 104 mg/kg, and its distribution was investigated over the period 30 minutes to 6 days post-dose. Ethoxyquin was found to be distributed throughout most tissues and the blood at 30 minutes post-dose. The highest radioactivity was

seen in the liver and kidneys (24 hours), gastrointestinal tract, and adipose tissue. This supports the previous suggestion that ethoxyquin metabolism occurs primarily in the liver and kidneys [Wilson et al 1959]. The hepatic peak was seen 8 hours post dose, at 24 and 48 hours levels of ethoxyquin were still raised in the kidney medulla, gastrointestinal tract and blood. Residues of ethoxyquin and its metabolites were still present after 6 days in some tissues (including the kidney cortex and the blood), supporting the proposed turnover time of 10 days for a dose of ethoxyquin [Wilson & DeEds 1959]. Skaare & Nafstaad [1979] also mentioned that ultrastructural effects were seen in the liver (proliferation of SER, dilated cisternae of ER, disorganisation of mitochondrial enzymes) following a single oral dose of 500 mg/kg ethoxyquin in a previous study [Nafstaad & Skaare 1978] and that ethoxyquin had previously been reported to be nephrotoxic at high doses by Wilson & DeEds [1959].

The first long-term studies with ethoxyquin [Rudra et al 1974] investigated the effects of ethoxyquin and vitamin E in the diet on food intake and body weight gain over 500 days, using male weanling Wistar rats. Ethoxyquin was given in arachis oil in the diet at a concentration of 0.5%. Decreases in body weight were noted, beginning after 280 days of treatment. These were reported to probably reflect a toxic response since 4 of 5 rats died with severe kidney damage. However, the nature of this kidney damage was not discussed. Rudra et al [1974] reported that no such damage was seen in mice treated with ethoxyquin and proposed, as Wilson & DeEds had earlier [1959], that there were interspecies difference in susceptibility, rats being more sensitive. Rudra and co-workers [1974] suggested that further work was therefore necessary to evaluate the hazard potential to domestic animals and man.

Further concerns regarding the safety of ethoxyquin were raised by McIntosh et al [1986] and Hernandez et al [1993]. McIntosh and co-workers [1986] suggested it was not necessarily

safe as a feed or diet additive, following acute intoxication of marmosets and rats fed high concentrations of dietary ethoxyquin. Due to an error, marmosets had been given ten times the normal amount of ethoxyquin in their food (800 p.p.m., or 0.08%), leading to the deaths of 30 marmosets in 3 weeks. On examination, these marmosets were found to have centrilobular giant cells in the liver and haemolytic anaemia. McIntosh and co-workers [1986] therefore tested the toxicity of this level of ethoxyquin in rats, and reported decreased body weight but no pathological changes in liver, kidneys or blood. Hernandez et al [1993], using renal cortical slices from male rats fed ethoxyquin, found that it inhibited the uptake of organic anion and cation tubular secretion. They suggested that the mechanism for this involved decreased ATPase activity and interference with the energy supply required, and suggested avoiding the exposure of animals to high ethoxyquin concentrations.

4.1.3: "Protective" potential of ethoxyquin

Synthetic antioxidants, including ethoxyquin, have been investigated in toxicological and pharmaceutical research due to their potential, by virtue of their peroxide and free radical-scavenging properties, to protect against chemical carcinogens in experimental animals. However, it has proven difficult to establish whether antioxidants are useful for this purpose as they have been shown to prevent some cancers but promote the development of others [Kahl 1984, Rao et al 1984, Tsuda et al 1984a & b, Manson et al 1987], ethoxyquin being a typical example of this.

It has generally been reported that ethoxyquin helps to protect against liver neoplasia in rats induced by various agents; Tsuda et al [1984a & b] reported that ethoxyquin, at a concentration of 0.8% in the diet, inhibited hepatocellular carcinoma and reduced the number of hyperplastic nodules normally seen with N-ethyl-N-hydroxyethylnitrosamine. Ethoxyquin

has also been shown to protect against hepatic tumours induced by ciprofibrate [Rao et al 1984] and prevent aflatoxin B₁- induced tumours [Manson et al 1987] at a concentration of 0.5% in the diet. It has also been reported to protect against the toxicity of carbon tetrachloride, dimethylnitrosamine and benzopyrene [Kahl 1984].

However, it has also been reported that ethoxyquin enhances the appearance of cancer in the kidney in some of these studies. Ethoxyquin was found to significantly increase the incidence of renal cell adenoma and preneoplastic kidney lesions [Tsuda et al 1984a & b] and ethoxyquin alone induced preneoplastic changes in the kidney cortex, possibly exerting a carcinogenic effect [Manson et al 1987]. Ethoxyquin has also been cited as a possible bladder cancer promoter [Shibata et al 1991].

4.1.4: Biochemical action of ethoxyquin

Kahl [1984] reported that although increasing attention had been given to antioxidants as potential protecting agents against carcinogens, ethoxyquin was rarely included in biochemical studies of antioxidants. He therefore investigated the biochemical action of ethoxyquin. He reported increases in liver and kidney weights, increased enzyme induction of liver and some kidney enzymes (cytochrome P450 and NADPH:quinone reductase in the kidney), and increased endoplasmic reticulum (ER) in the liver. The latter observation supports previous ultrastructural observations in the liver following ethoxyquin treatment [Nafstaad & Skaare 1978].

Ethoxyquin was also found to interfere with the immune response, inhibit lipid peroxidation in biological membranes, enhance microsomal hydrogen peroxide production, and inhibit drug co-oxidation during prostaglandin synthesis [Kahl 1984]. The effects of ethoxyquin on

prostaglandin production and cyclo-oxygenase and lipoxygenase activity have been investigated, in renal medullary slices and homogenate respectively, and compared with the effects of NSAIDS, particularly indomethacin [Zenser & Davies 1978, Bach & Bridges 1984, Miyazawa et al 1985]. Ethoxyquin, in common with NSAIDS, was shown to inhibit prostaglandin synthesis (particularly PGE₂) and cyclo-oxygenase activity. Ethoxyquin was in fact found to be a more potent inhibitor of these than indomethacin, and also slightly inhibited lipoxygenase activity. The above actions of NSAIDS are implicated in the development of RPN, as discussed in Chapter 1 of this thesis.

4.1.5: Ethoxyquin and renal papillary necrosis

Ethoxyquin was first reported to induce RPN in studies by Hard & Neal [1990 & 1992]. Early toxicity studies with ethoxyquin in Fischer F344 rats showed inflammatory cortical changes, accelerated chronic progressive nephropathy and severe proximal tubule hypertrophy suggestive of preneoplastic change (supporting earlier suggestions, mentioned above, that it acts as a cancer promoter in the kidney). To clarify this, they fed ethoxyquin to rats (0.5% in the diet) over periods ranging from 4 weeks to 20 months [1990]. They found, to their surprise, that the primary lesion in male rats was RPN, first apparent at 4 weeks, followed by pyelonephritis in the cortex. Urothelial hyperplasia was also a prominent lesion in males after 24 weeks of treatment. Female rats did not show any signs of RPN until 24 weeks, and even then only interstitial degeneration was observed. They therefore reported that they had demonstrated a previously unrecorded lesion with ethoxyquin (RPN). The early work by Wilson & DeEds [1959] reported kidney damage following ethoxyquin treatment but details of this damage were not given; it is possible that this may have included RPN.

The second study by Hard & Neal [1992] was a sequential study in Fischer rats with ethoxyquin treatment for 4 weeks to 18 months, which also reported RPN as the primary lesion in male rats. After 4 weeks of treatment, 3 of 8 males showed interstitial degeneration. This progressed to RPN after 14 weeks and complete necrosis after 24 weeks with associated pyelonephritis. They also reported capillary changes. A marked sex difference was again demonstrated, with female rats never progressing beyond interstitial degeneration.

Manson et al [1992] reported that the degree of ethoxyquin-induced damage was age-dependent and sex-dependent (supporting the findings of Hard & Neal [1990 & 1992]). They also used Fischer F344 rats and ethoxyquin at 0.5% in the diet, and compared weanling (3 weeks old) and adult (8 weeks old) males with adult females. They reported cortical damage in males which was similar in both age groups, but male weanling rats also had extensive RPN after 20 weeks of treatment, with increased proteinuria. Proteinuria was also reported in control male rats, but this was greatly exacerbated by ethoxyquin treatment. Female adult rats were much less susceptible to the effects of ethoxyquin, as reported by Hard & Neal [1990 & 1992]. Manson et al [1992] therefore concluded that the extent of damage to the papilla was influenced by the initial age of the animal as well as the duration of treatment. Manson & co-workers [1992] (and Hard & Neal [1990 & 1992]) reported that the underlying cause of RPN induced with ethoxyquin was unknown, but recommended not using weanling rats in any carcinogenicity studies with ethoxyquin since their susceptibility to RPN could affect such studies.

4.1.6: Aims of the studies described in this chapter

The previous studies with ethoxyquin which investigated RPN involved dosing for at least 4 weeks, with ethoxyquin given in the diet at a concentration of 0.5%. Detailed investigations regarding the nature of the RPN had not previously been reported. The initial aim of the present experiments was therefore to develop a dose regime with ethoxyquin whereby RPN could be reliably and reproducibly induced over a shorter time frame, to allow subsequent assessment of ethoxyquin as a model for RPN. Weanling male rats were predominantly used in the experiments described in this chapter due to their reported susceptibility to RPN with ethoxyquin [Hard & Neal 1990 & 1992, Manson et al 1992]. Oral gavage was used as a dose route rather than dietary administration as this would allow a more accurate indication of actual doses given to the animals and would make dose-ranging studies easier to perform.

Another aim of these experiments was to further assess the techniques identified by the experiments in the previous chapter as potentially useful for studying RPN (SEM and urinary sediments, including urine analysis). This would then allow comparison of the results of these techniques using 2 established models of RPN (BEA and indomethacin) with the results obtained using a new model, ethoxyquin.

For ethoxyquin to have potential as a model for RPN, it was also necessary to investigate whether a dose regime could be developed which would produce only RPN, with no cortical damage and little or no extra-renal toxicity. Rosner [1976] has emphasised the importance of looking at all organs (not just the kidney) to evaluate the real picture of damage with a compound. In investigating this, a more detailed picture of any extra-renal effects of ethoxyquin would be obtained, which could be compared with previous work using ethoxyquin and provide more information regarding the effects of this compound.

Following establishment of an ethoxyquin dose regime which reproducibly induced RPN, it was also intended to investigate the development of the lesion using transmission electron microscopy (TEM), with the aims of identifying the primary cell type affected and the course of events involved. This would then allow comparison of RPN induced by ethoxyquin with that induced by other experimental models and RPN reported in man, and the possible mechanisms involved.

4.2 MATERIALS & METHODS

4.2.1: Materials

4.2.1.1: Chemicals and suppliers

Histological chemicals and materials were identical to those described in 3.2.1.1. Electron microscopy fixatives, chemicals, specimen stubs and grids were obtained from Agar Scientific Ltd, Essex. Dyes used in the Toluidine blue and Methylene Blue/Azure II staining methods were obtained from BDH Merck. Anaesthetics and perfusion solutions were as described in 3.2.1.1. Ethoxyquin and corn oil were obtained from Sigma-Aldrich Ltd., Dimethyl sulphoxide (DMSO) was obtained from BDH Merck.

The Multistix 10SG dipstick tests used in urine analysis, all assay kits used in the clinical chemistry investigations and staining kits used in the haematology investigations were obtained from Bayer Diagnostics, Bayer PLC, Hampshire.

4.2.1.2: Animals, housing conditions and dosing regimes

All strains of rat were obtained from Charles River (UK) Ltd.; the strains used in each experimental phase are detailed in the appropriate methods section. Animals were housed at a temperature of $21 \pm 2^{\circ}\text{C}$, relative humidity of $55 \pm 10\%$ and 12 hours light/12 hours dark lighting. Animals were fed with a standard certified RMM rodent diet, both this and water were provided *ad libitum*. The practical work carried out in this chapter was separated over several experiments for logistical reasons; the numbers of animals used in each experiment are detailed in Table 4.1.

Dosing was by oral gavage (oral intubation). The dosing regimes and vehicles used for ethoxyquin are detailed in the methods sections. Unless otherwise specified, all animals were killed 24 hours

Table 4.1: Number of animals used in ethoxyquin experiments and numbers used for each technique

Technique	Dose	Ranging	Studies	Total Numbers	Main Studies			Total Numbers
Histology	13 (13)	40 (40)	84 (20)	137 (73)	20 (10)	-	10 (10)	30 (20)
Urine sediment	-	-	-	-	20 (10)	20 (20)	10 (10)	50 (40)
Volumes noted					Yes	Yes	Yes	50 (40)
Urine Chemistry	-	-	-	-	20 (10)	20 (20)	10 (10)	50 (40)
SEM	-	-	-	-	20 (10)	-	-	20 (10)
Secondary histology					Yes			20 (10)
TEM	-	-	-	-	12 (6)	20 (20)	-	32 (26)
Clinical chemistry	-	-	-	-	-	-	10 (10)	10 (10)
Haematology	-	-	-	-	-	-	10 (10)	10 (10)
EXPERIMENT	E	F	G	-	H	I	J	-

The number of treated animals used for each technique is given first, the number of control animals is given in brackets. The numbers used are split into columns for each separate experiment conducted. Experiments are coded E-J at the bottom of the table.

after the last dosing by an intra-peritoneal overdose of sodium pentobarbitone ("Sagatal", 0.2mL/100g body weight).

4.2.1.3: Grading criteria for histological and electron microscopy (EM) assessment

The grading system used for assessment of renal papillary necrosis (RPN) is detailed in Table 4.2a.

Table 4.2a: Grading criteria for assessment of RPN

Grade	Histological appearance	EM appearance
Degeneration	Pre-necrotic change; not seen at histological level (classed as NAD)	Distortion/derangement of cells or organelles, vacuolation of cells & possible degeneration- pre-necrotic changes.
Minimal RPN	Very slight damage to small area of papilla - damage to interstitial cells only	As for Histology
Mild RPN	Slight damage to papilla tip- loss of interstitial cells, possible early damage to cells in some loops of Henle and/or capillaries (vacuolation of cells), preservation of most ducts & tubules	As for Histology
Moderate RPN	Loss of interstitial cells in area of necrosis, derangement of some tubules (mainly loops of Henle and capillaries) but preservation of papilla structure	As for Histology
Marked RPN	Destruction of papilla structure in area of damage; loss of interstitium, tubules and ducts (outline only remaining)	As for Histology

Key: RPN = renal papillary necrosis EM = electron microscopy
NAD = no abnormalities detected

Renal cortical necrosis was graded as detailed in Table 4.2b.

Table 4.2b: Grading criteria for cortical necrosis

Grade	Histological appearance
Mild	small focal areas with tubular necrosis, but structure preserved.
Moderate	larger area of necrosis with some derangement of tubular structures.
Marked	destruction of tubular structures within area of necrosis, more extensive area affected than in moderate, with a diffuse band of necrosis through the cortex.

4.2.2: Development of Ethoxyquin as a model for RPN

For all studies involving the development and assessment of ethoxyquin as a model for RPN, standard histological evaluation was used to assess all kidney samples for the presence of RPN. The methods used are detailed in Chapter 3, Section 3.2.2.

4.2.2.1: Dose-ranging studies with DMSO.

The first stage of investigating ethoxyquin as a model for RPN was to assess the tolerability of various doses in rats, when given by oral intubation in dimethyl sulphoxide (DMSO). This was to gain information for use in future studies with ethoxyquin to evaluate its use as a papillotxin.

Initially, female (6 weeks old, 150-200g) and male (3-4 weeks old, 75-100g) Sprague-Dawley rats were given a single dose of ethoxyquin by oral intubation at one of the following doses: 100mg/kg; 200mg/kg; 300mg/kg; 400mg/kg; 500mg/kg and 600mg/kg. All animals were killed 48 hours post-dose and kidneys were removed and processed for histological assessment using a Bayer VIP processor as in 3.2.2.

Following the initial assessment, a second phase of work was carried out to further assess the dose tolerance with ethoxyquin and to investigate the effects of 2 different dose levels (600mg/kg and 800 mg/kg) after both a single dose and 5 daily doses. For this second assessment, 20 male Sprague-Dawley rats (3-4 weeks old, 75-100g) were dosed with either vehicle (n=2/control group), or ethoxyquin at a dose of 600mg/kg or 800mg/kg (n=4/treatment group). Ten animals were given a single dose of the relevant material, the other ten received 5 daily doses. Animals were killed 24 hours after completion of dosing and both kidneys removed and processed for histological evaluation.

4.2.2.2: Change of dosing vehicle and investigation of rat strain for model

Following tolerance studies with concentrations of up to 800mg/kg ethoxyquin (in DMSO), with dosing for up to 5 days, the nephrotoxic potential of ethoxyquin was further investigated by assessing the effects of twice-daily dosing (totalling 800 mg/kg/day) for up to 28 days, using corn oil as a vehicle instead of DMSO. It was felt that DMSO was an undesirable vehicle for this length of study due to its noxious smell.

Two strains of male rat were used in this study to allow inter-strain comparison of any renal effects; male Sprague-Dawley and Fischer F344 rats (3-4 weeks of age, 75-100g) were dosed twice daily by oral intubation with either corn oil vehicle (2 animals/control group/timepoint) or 400mg/kg ethoxyquin (4 animals/treatment group/timepoint).

Scheduled timepoints for termination were Days 2, 7, 14 and 28. Kidneys were taken at termination from all animals, fixed in 10% neutral buffered formalin (NBF) and processed to paraffin block as in

3.2.2. Sections were cut and stained with H&E for histological assessment. Any macroscopic abnormalities in other organs were also sampled and fixed in 10% NBF for possible histological

examination. All kidney sections were assessed for renal effects and any damage graded for both papilla and cortex as described in section 4.2.1.3.

4.2.2.3: Establishment of optimum dosing regime with corn oil vehicle

Based on the results from the previous stages of model development, the tolerance of Sprague-Dawley rats to ethoxyquin was further assessed using a range of dose concentrations and dosing periods. Male Sprague-Dawley rats (3-4 weeks of age, 75-100g) were dosed either once or twice daily for up to 28 days with either corn oil vehicle or ethoxyquin to give a total dose of 100, 200, 400, 500 or 600mg/kg/day.

Scheduled timepoints for termination were Days 3, 8, 15 and 29 (24h after last dose). Kidneys were taken at termination from all animals, fixed in 10% NBF and processed to paraffin block. Any macroscopic abnormalities present at termination were also sampled, fixed and processed. Sections (4µm) were cut and stained with Haematoxylin & Eosin for histological assessment.

All kidney sections were assessed for renal effects, without knowledge of treatment group, and any papillary damage graded as detailed in section 4.2.1.3. A “cumulative score” of papillary necrosis was also determined for each group: the grade of papillary necrosis seen in each animal was given a “score”, as described in Table 4.3 below, and the cumulative score for each dose group calculated. This was to help distinguish between the grades of damage seen rather than just recording the incidence of necrosis in each group, and was used to help determine the optimum dose regime for ethoxyquin.

Table 4.3: Scoring system used to distinguish between grades of necrosis seen within a dose group

Grading	Score
Normal	0
Minimal RPN	1
Mild RPN	2
Moderate RPN	3

Any effects on the kidney cortex were also noted, as were microscopic observations on any tissues sampled with macroscopic findings.

4.2.2.4: Dose regimen used in further work with ethoxyquin

Following the above work detailing the development of ethoxyquin as a model for RPN, the dose regimen used in all further studies with ethoxyquin was 500mg/kg/day in corn oil, given as a once daily dose by oral gavage.

4.2.3: Urinary sediments

Urine samples were collected on ice overnight (16.30 to 8.30h) after 1, 2, 3, 4 or 7 daily doses of ethoxyquin (Days 2, 3, 4, 5 and 8). Animals were housed in MS&D metabolism cages for urine collection. Urine volumes were measured with a measuring cylinder and fixed urinary sediments were prepared as detailed in Chapter 3.2.3.

4.2.4: Perfusion methods

The kidneys of some animals were perfuse-fixed for optimal preservation of cells and tubules where Transmission Electron Microscopy investigation was performed (4.2.6 below). Under deep

anaesthesia, kidneys were perfuse-fixed with modified Karnovsky's solution after a saline wash via whole body perfusion, using constant head of pressure apparatus set at 110 mm Hg (as detailed in 3.2.4).

4.2.5: SEM and correlative histology

Kidneys were taken at termination from animals after treatment with ethoxyquin or vehicle (controls) for 2 and 7 days, fixed in 10% neutral buffered formalin (NBF) and the papillae processed for SEM assessment using a Philips XL20 microscope, as described earlier (3.2.6). Any alterations in the papilla structure were noted.

Following this assessment, all papillae and kidney cortex samples from each animal were processed through to paraffin block and H&E sections were prepared to allow comparative histological assessment. All kidney sections were assessed for renal effects without knowledge of treatment group, and any papillary necrosis graded as detailed in Table 4.2 (Section 4.2.1.3). Any effects on the kidney cortex were also noted, as were microscopic observations on any tissues sampled with macroscopic findings at necropsy.

4.2.6: Time-course of lesion development using transmission electron microscopy (TEM)

Animals were killed 24 hours after 1, 2, 3, 4, or 7 daily doses of ethoxyquin (i.e. on Days 2, 3, 4, 5 and 8). Following perfusion fixation as detailed in 4.2.4, papillae from all animals were dissected out and immersed in Karnovsky's fixative for a further hour, then post-fixed in 1% osmium tetroxide in 0.1M phosphate buffer pH 7.4 for one hour. Samples were then dehydrated through graded alcohols to 1,2 epoxypropane (25, 50, 75 and 2 x 100% alcohol, 15 mins each, 30 mins 1,2 epoxypropane), and infiltrated in CY212 araldite resin overnight before embedding and polymerisation in araldite blocks (48 hours at 60°C).

Semi-thin (1 micron) sections were cut and stained with either Methylene blue/Azure II or Toluidine Blue for light microscopy assessment; ultrathin (60- 90 nm) sections were cut and stained with uranyl acetate and lead citrate for conventional TEM assessment using a Philips CM10 electron microscope operating at 100kV. Any effects on cell architecture and ultrastructure were noted, and any RPN was graded as detailed in the EM portion of Table 4.2.

Samples of papillae were also removed fresh, 24 hours after the last dose, from some animals treated for 2 and 7 days with ethoxyquin and immerse-fixed for 2 hours in modified Karnovsky's fixative before processing as above to resin block. Sections were cut and assessed as above for perfuse-fixed samples. This was to allow comparison of perfuse- and immerse-fixed papillae for tissue preservation.

4.2.7: Urine analysis

Basic urine analysis was performed on all samples collected for urine sediment preparation (4.2.3). This analysis consisted of semi-quantitative Clinitek "dipstick" analysis of the following parameters: glucose, bilirubin, ketones, specific gravity, blood, pH, protein and urobilinogen. Colour changes in Multistix 10SG dipsticks were analysed by spectrophotometry using an Ames Clinitek 200 analyser, according to the manufacturer's instructions.

4.2.8: Clinical chemistry & haematology

Samples of blood were taken for analysis from animals at termination 24 hours after 2 and 7 daily doses of ethoxyquin.

4.2.8.1; Clinical chemistry

At necropsy, blood samples (1.5mL) were obtained from all animals via the abdominal aorta into tubes containing lithium heparin (32 IU/mL) for the determination of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, total protein, albumin, globulin, glucose, total bilirubin, conjugated bilirubin, cholesterol, triglycerides, urea, creatinine, sodium, potassium, chloride, calcium and phosphate. Determination was carried out on a Bayer Technicon Axon analyser, using the recommended Bayer Diagnostics kit for each parameter. Statistical analysis methods were applied as detailed in 4.2.10.

4.2.8.2; Haematology

At necropsy, blood samples (0.7mL) were obtained from all animals into tubes containing EDTA for determination of haemoglobin and platelets using a Sysmex E2500 automated haematology analyser. Haemoglobin was measured by spectrophotometry at 535nm, after conversion to the stable cyanmethaemoglobin, and blood cells and platelets were counted by detecting a difference in conductivity between the particles and the diluent in which they were suspended. Statistical analysis methods were applied as detailed in 4.2.10. Morphological assessment of red blood cells and platelets was also performed using stained preparations (Wright's stain, Bayer Diagnostics) and light microscopy.

4.2.9: Necropsy & histological assessment

Animals given 2 and 7 daily doses of ethoxyquin underwent full necropsy following blood sampling to identify any non-renal effects of ethoxyquin. All major tissues (brain, spinal cord, liver, kidneys, adrenal glands, heart, lungs, stomach, small and large intestines, spleen, pancreas, testes, thymus, lymph nodes, skeletal muscle, bone, skin, thyroid gland, pituitary and salivary glands) were preserved in 10% NBF and samples of each were processed to paraffin block for histological

assessment as detailed previously (3.2.2). Any macroscopic abnormalities noted at necropsy were also processed for histological assessment.

4.2.10: Statistical analysis

Bartlett's test of homogeneity was carried out before statistical analysis to identify any "outliers" in the clinical chemistry and haematology results. Dunnett's modified T-test was then applied to test the significance of any differences in results between control and treated samples.

4.3 RESULTS

4.3.1: Development of ethoxyquin as a model for renal papillary necrosis

4.3.1.1: Dose-ranging studies with DMSO

All doses of ethoxyquin (up to 800 mg/kg) and both dosing periods (single dose and 5 daily doses) were well tolerated. No difference was noted between Sprague-Dawley males and females in the initial assessment, so males were selected for future studies with ethoxyquin. The only clinical sign, seen in both control and treated animals that were dosed for 5 days, was brown staining of tray paper after 2 doses, which did not recur. Upon necropsy, all top dose animals (800mg/kg) treated for 5 days showed pale red discolouration of the intestines. Histological assessment of H&E-stained kidney sections did not show any obvious signs of treatment-induced lesions, although kidneys from 3 of 4 top dose animals (800mg/kg) did show possible degenerative changes after 5 days treatment, consisting of shrunken nuclei and foamy cytoplasm.

4.3.1.2: Change of dosing vehicle and investigation of rat strain

In this experiment the vehicle had been changed from DMSO to corn oil and the maximum dose of 800 mg/kg ethoxyquin from the previous experiment was used; this was split into 2 daily doses of 400 mg/kg to determine whether twice daily dosing increased the nephrotoxicity of ethoxyquin. Two strains of male rat were investigated to assess the relative sensitivity of these and allow inter-strain comparison of any renal effects. Following scheduled termination of Day 2 animals, all remaining animals were killed on Day 4 due to unexpected, unacceptable clinical signs in all treated animals (marked lethargy or moribund condition), none of which had been apparent in animals given the same dose of ethoxyquin in DMSO. Some animals were killed on day 2 (2 Fischer rats) and day 3 (5 Sprague-Dawley and 10 Fischer rats) in a moribund condition. Fischer rats showed more severe clinical signs than Sprague-Dawley rats.

Abnormalities related to clinical signs

Macroscopic examination of animals revealed stomach ulcers, apparent as small white areas which in 3 cases also had dark red foci. Pale livers and darkened intestines were also in some treated animals (Table 4.4, below). The site of ulceration within the stomach differed between strains; the non-glandular region was affected in Sprague-Dawley rats (11/20) whereas in Fischer rats the pylorus area of the glandular region was affected (7/20). The degree of ulceration was more severe in Fischer rats, in two animals the ulcers had perforated. Histological assessment of the stomachs confirmed the macroscopic observations (Fig. 4.1), showing inflammation and ulceration of the gastric mucosa; this had penetrated the stomach wall in those animals where perforated stomachs were noted at necropsy. No microscopic abnormalities were seen in the livers and intestines that had been sampled with macroscopic findings (pale livers, dark intestines).

Table 4.4: Clinically-related findings from ethoxyquin-treated animals: incidence table

Macroscopic Finding	Fischer Rats	Sprague-Dawley Rats
STOMACH: Normal	13/20	9/20
Ulcerated	5/20	11/20
Perforated	2/20	0/20
LIVER: Normal	14/20	17/20
Pale	6/20	3/20

Figure 4.1: Stomach lesions induced with ethoxyquin treatment

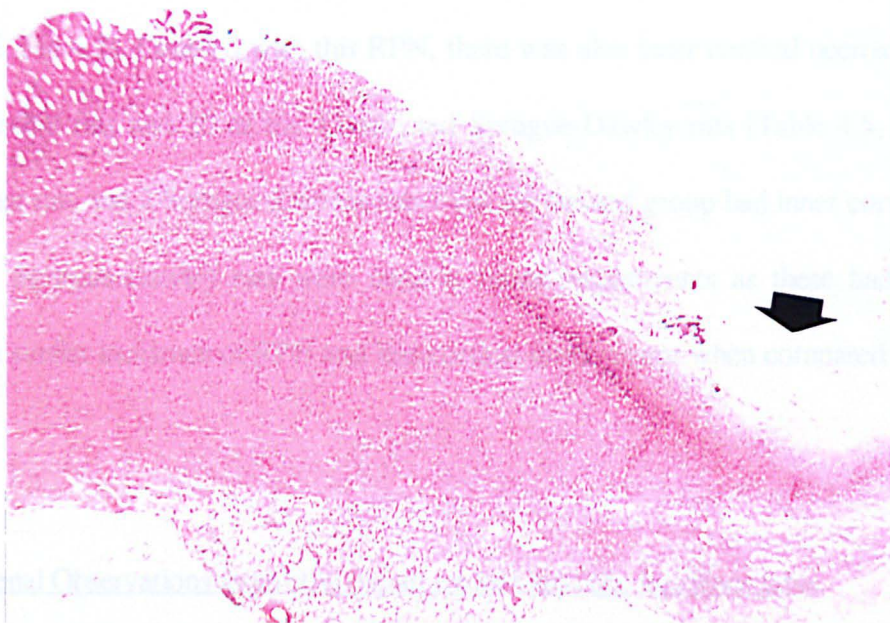


Figure 4.1a: Lesions induced in Fischer F344 rat: ulceration of glandular stomach (arrow). H&E x 50.



Figure 4.1b: Lesions induced in Sprague-Dawley rat: ulceration of non- glandular region of stomach (arrow). H&E x 50.

Renal observations

Histological assessment of kidneys revealed RPN in 9/20 Fischer rats and 11/20 Sprague-Dawley rats. This RPN was more focal in Sprague-Dawley rats but of similar severity to that seen in Fischer rats (Table 4.5, Fig 4.2). Coupled with this RPN, there was also inner cortical necrosis in 8 of the 10 affected Fischer rats and 10 of the 11 affected Sprague-Dawley rats (Table 4.5, Fig. 4.3). A further 6 Fischer rats and 2 Sprague-Dawley rats from the treated group had inner cortical necrosis without RPN. Sprague-Dawley rats were used in future experiments as these had less severe clinical signs, a similar incidence of RPN and less cortical involvement when compared with Fischer rats.

Table 4.5: Renal Observations from ethoxyquin-treated animals: incidence table

Histology Observations	Fischer rats	Sprague-Dawley rats
PAPILLA: Normal	11/20	9/20
<u>RPN</u> ; Minimal	1/20	4/20
Mild	3/20	2/20
Marked	5/20	5/20
Total with RPN	9/20	11/20
CORTEX: Normal	6/20	8/20
<u>Necrosis</u> ; mild	9/20	9/20
moderate	4/20	2/20
marked	1/20	1/20
Total with cortical changes	14/20	12/20

Figure 4.2: RPN induced in Fischer and Sprague-Dawley rats with ethoxyquin

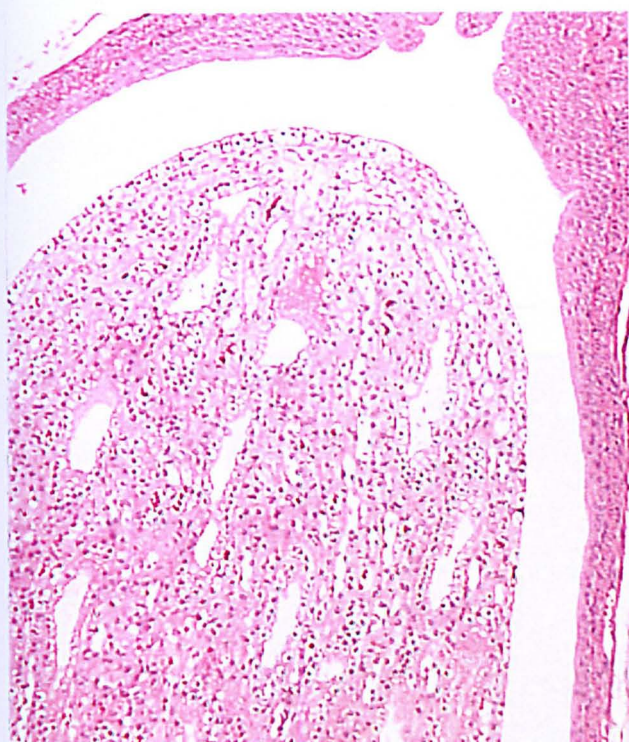


Figure 4.2a: Fischer rat: control kidney.
H&E x 100

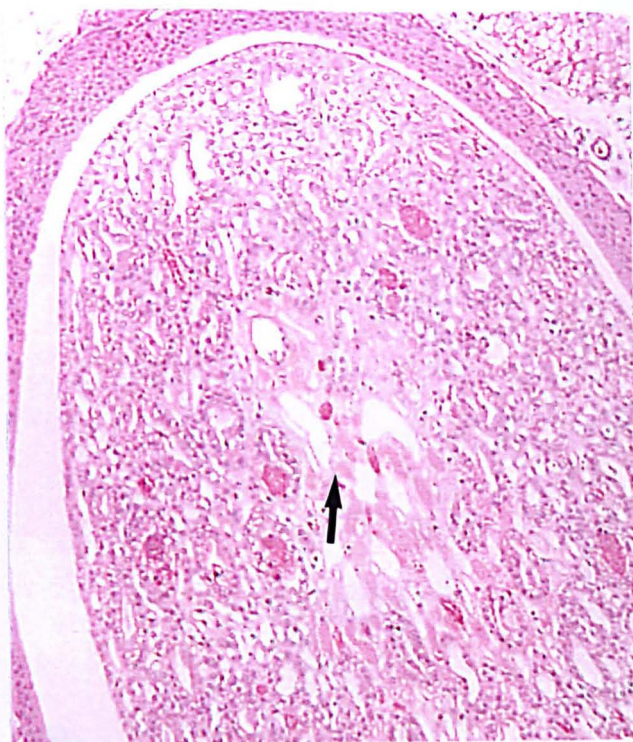


Figure 4.2b: Fischer rat: Treated kidney.
Note area of focal necrosis (arrow).
H&E x 100



Figure 4.2c: Sprague-Dawley rat: control
kidney. H&E x 100



Figure 4.2d: Sprague-Dawley rat: Treated
kidney. Note area of focal necrosis (arrow),
which is larger than Fischer lesion.
H&E x 100

Figure 4.3: Cortical necrosis induced with ethoxyquin

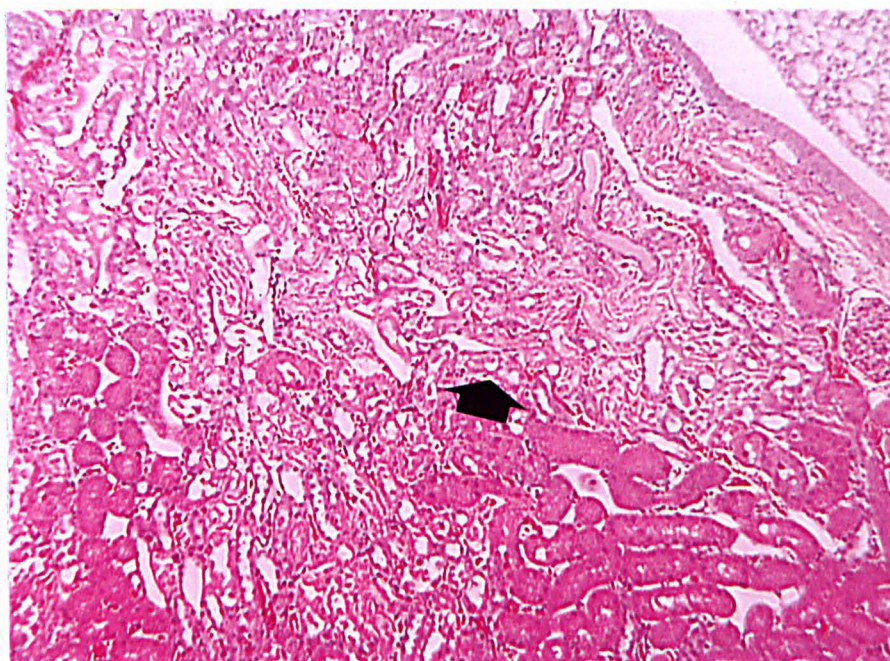


Figure 4.3: Marked cortical lesion induced in Fischer F344 rat following ethoxyquin treatment. Note area of necrosis (arrow). H&E x 100.
H&E x 100.

4.3.1.3; Establishment of optimum dosing regime with corn oil vehicle

With doses of up to 600 mg/kg ethoxyquin given in corn oil, all but 2 of the animals survived to the scheduled termination points with no adverse clinical signs. No difference was seen between animals given ethoxyquin as once-daily or twice-daily doses. One animal given 500mg/kg/day was killed in a moribund condition on Day 2 of treatment, and one given 600 mg/mg/day was found dead on Day 21. Damp, brown staining fur around the mouth/throat was noted in all treated animals after each dosing, but this regressed between doses.

Extra-renal observations

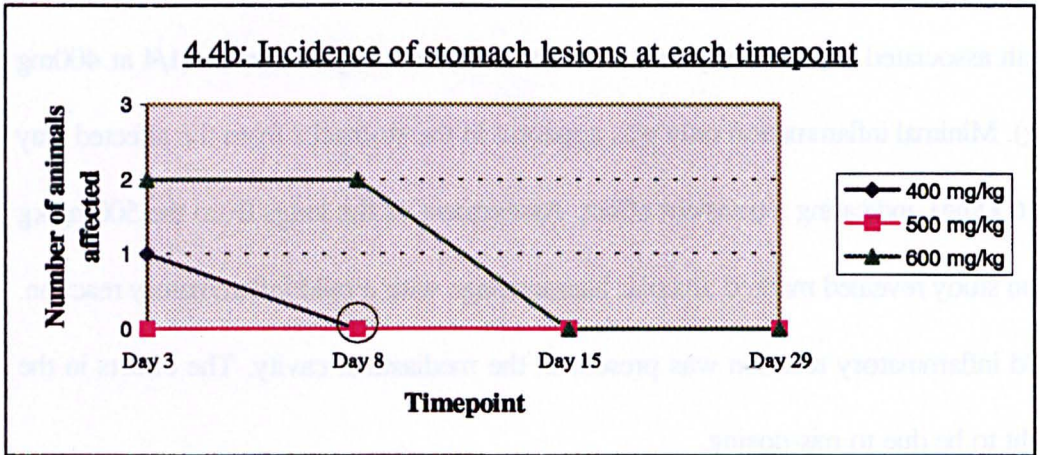
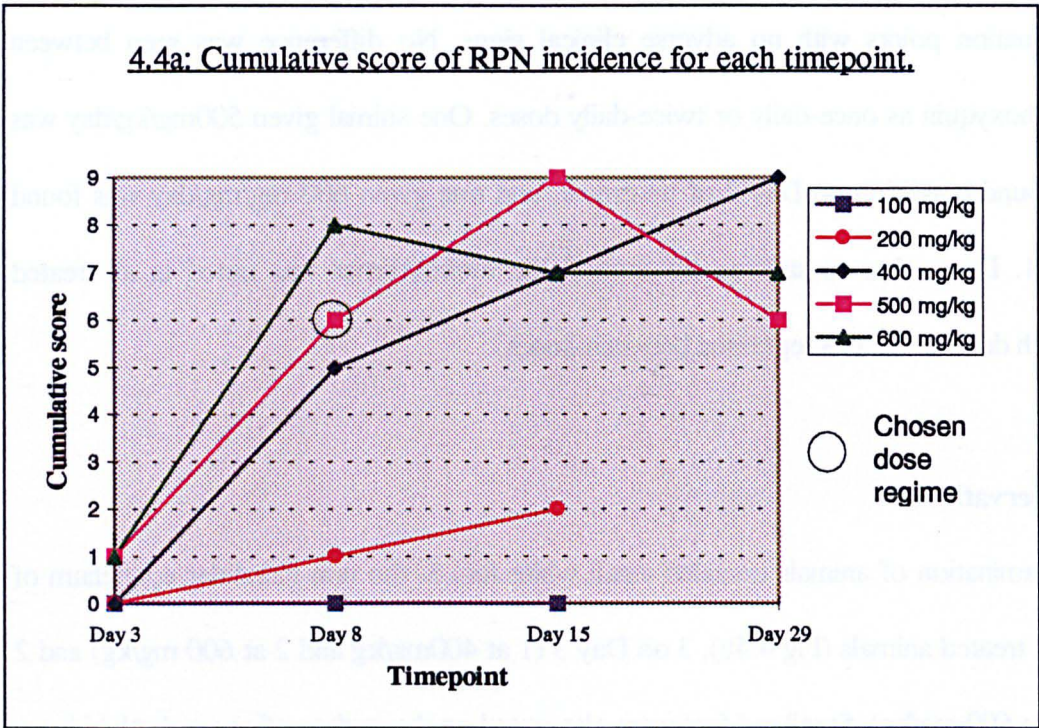
Macroscopic examination of animals revealed small white foci in the non-glandular epithelium of the stomach in 5 treated animals (Fig 4.4b); 3 on Day 3 (1 at 400mg/kg and 2 at 600 mg/kg) and 2 on Day 8 (both at 600 mg/kg). Small red foci were also noted on the surface of one or both kidneys from 4 treated animals (3 at a dose of 100 mg/kg and 1 at 400 mg/kg ethoxyquin).

Histological assessment of the macroscopic stomach lesions revealed focal ulceration of the non-glandular area with associated inflammatory reaction in the affected Day 3 animals (1/4 at 400mg and 2/4 at 600mg). Minimal inflammation only was apparent in the stomachs from the affected Day 8 animals (2/4 at 600mg), indicating a transient effect. Assessment of the lungs from the 500mg/kg animal that died on study revealed marked alveolar haemorrhage with a mild inflammatory reaction. In addition, a mild inflammatory reaction was present in the mediastinal cavity. The effects in the lungs were thought to be due to mis-dosing.

Renal observations

Histological examination of kidneys identified RPN in some treated animals. The incidence of RPN increased with dose group and time (Table 4.6): on Day 3 only 2/12 animals were affected and only

Figure 4.4: Dose ranging ethoxyquin study; evaluation of optimum dose regime



at 500 and 600 mg/kg; by Day 8 half of the 400 mg/kg dose group and all animals at or above 500 mg/kg ethoxyquin were affected. By Day 15 the number of 400 mg/kg animals affected had risen to 6/8, with all animals affected in this dose group at day 29. Comparing the “cumulative scores” calculated for each group, this distinguished well between the extent of RPN seen in each dose group (Table 4.6 and Figure 4.4a).

The severity of RPN at each timepoint varied with all groups from minimal damage where only interstitial cells were affected to moderate RPN where derangement and necrosis of loops of Henle and capillaries was also evident (Table 4.6; Fig. 4.5). The kidneys of some 600 mg/kg animals also had minimal tubular degeneration and necrosis in the cortex on Days 15 and 29 (2/4 at each timepoint).

From these results, the dose regime chosen for all future ethoxyquin experiments was 500 mg/kg ethoxyquin in corn oil, since this induced RPN in all animals after 8 daily doses with minimal clinical signs (Fig. 4.4, chosen dose regime circled).

Table 4.6: Severity grading and incidence of RPN in treated animals

Timepoint and grading	100 mg/kg ethoxyquin	200 mg/kg	400 mg/kg	500 mg/kg	600mg/kg
Day 3					
Normal	4 (100%)	4 (100%)	8 (100%)	3 (75%)	3 (75%)
Minimal	0	0	0	1 (25%)	1 (25%)
Mild	0	0	0	0	0
Moderate	0	0	0	0	0
TOTAL number of animals	4	4	8	4	4
Cumulative score	0	0	0*	1	1
Day 8					
Normal	4 (100%)	3 (75%)	4 (50%)	0	0
Minimal	0	1 (25%)	0	2 (50%)	0
Mild	0	0	2 (25%)	2 (50%)	4 (50%)
Moderate	0	0	2 (25%)	0	0
TOTAL number of animals	4	4	8	4	4
Cumulative score	0	1	5*	6	8
Day 15					
Normal	4 (100%)	3 (75%)	2 (25%)	0	0
Minimal	0	0	1 (12.5%)	0	2 (50%)
Mild	0	1 (25%)	2 (25%)	3 (75%)	1 (25%)
Moderate	0	0	3 (47.5%)	1 (25%)	1 (25%)
TOTAL number of animals	4	4	8	4	4
Cumulative score	0	2	7*	9	7
Day 29					
Normal	-	-	0	0	0
Minimal	-	-	1 (25%)	2 (50%)	2 (50%)
Mild	-	-	1 (25%)	2 (50%)	1 (25%)
Moderate	-	-	2 (50%)	0	1 (25%)
TOTAL number of animals	0	0	4	4	4
Cumulative score	0	0	9	6	7

Percentage incidence for each grade at each timepoint is given in brackets where applicable.

Key: - = no animals dosed for this period of time at these dose levels.

* = cumulative score divided by 2 to account for increased group size at these timepoints.

Cumulative Score:

Necrosis grade	Score given
Normal	0
Minimal RPN	1
Mild RPN	2
Moderate RPN	3

Figure 4.5: Grades of RPN induced in Sprague-Dawley rats following ethoxyquin treatment

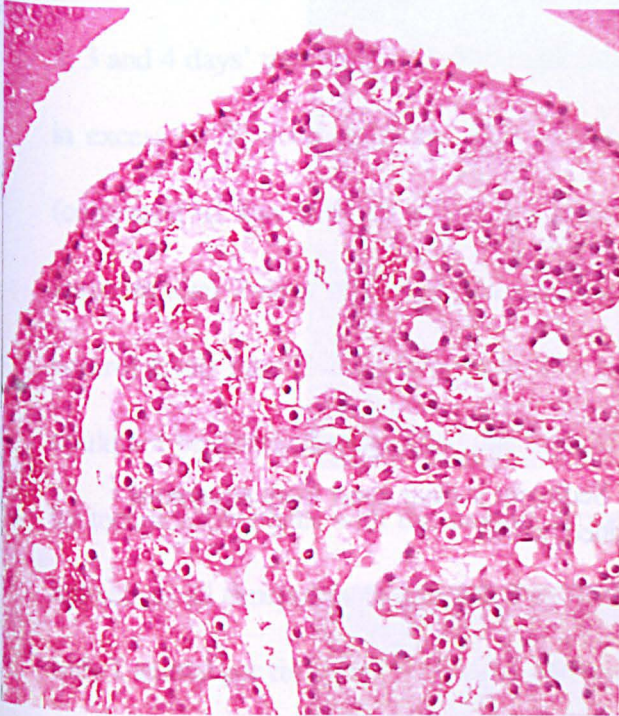


Figure 4.5a: Control kidney. H&E x 250

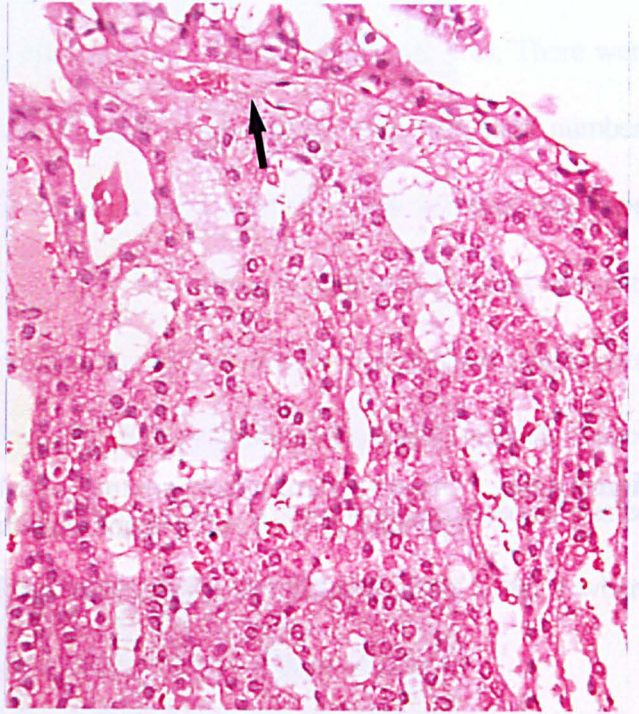


Figure 4.5b: Treated kidney: Minimal necrosis. Note loss of interstitial detail at tip (arrow). H&E x 250



Figure 4.5c: Mild Necrosis. Note loss of interstitium and necrosis of some tubules but outlines remaining (arrow). H&E x 250

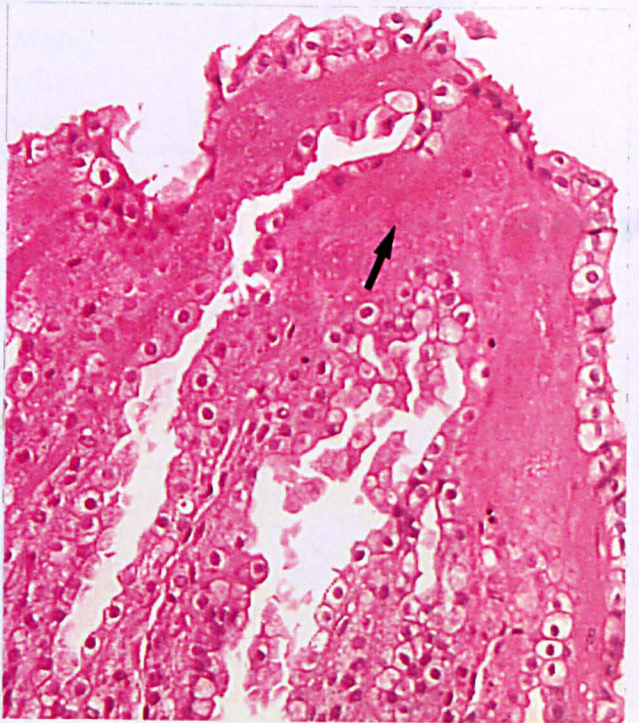


Figure 4.5d: Moderate necrosis. Note loss of structural detail in tip region (arrow). H&E x 250

Figure 4.6: Urinary sediment changes following ethoxyquin treatment

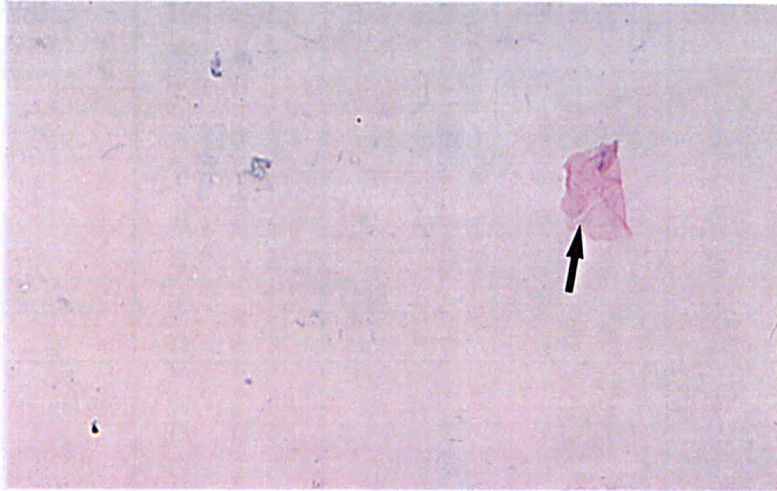


Figure 4.6a: Urinary sediment from control animal. Sediment is clear apart from squamous epithelial cell (arrow). Papanicolaou-stained, x 250.

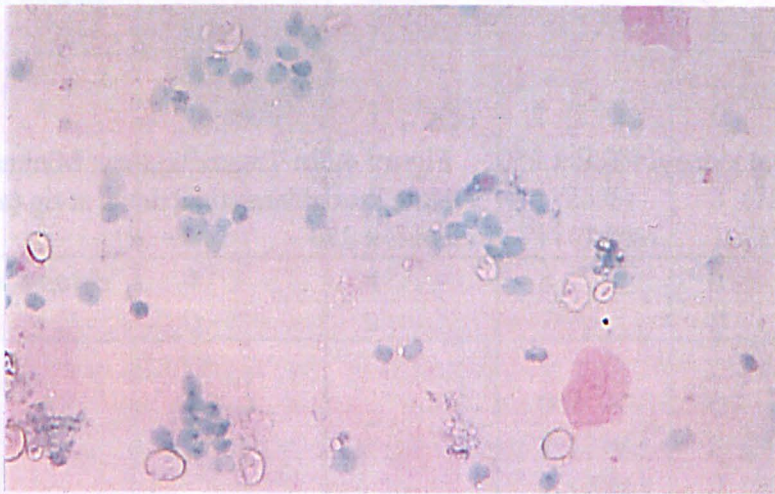


Figure 4.6b: Urinary sediment from ethoxyquin-treated animal at Day 3: Note large numbers of epithelial cells compared to control sediment. Papanicolaou-stained, x 250.

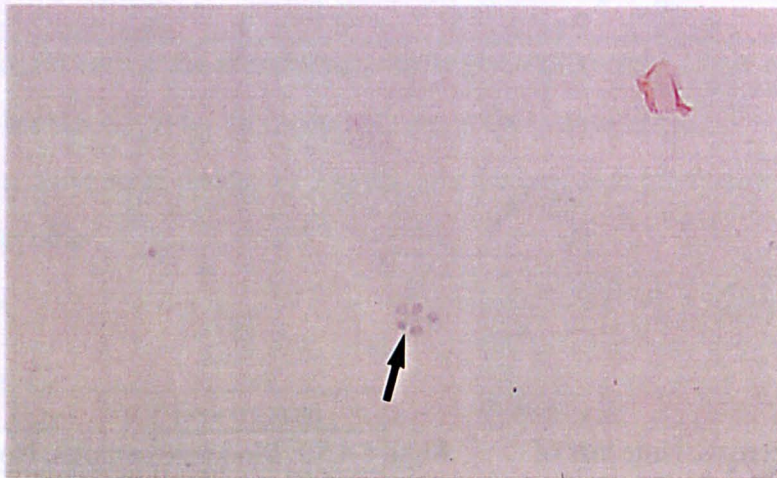


Figure 4.6c: Urinary sediment from ethoxyquin-treated animal at Day 8. Sediment appears similar to control animal apart from a small cluster of epithelial cells (arrow). Papanicolaou-stained, x 250.

4.3.2: Urinary Sediments

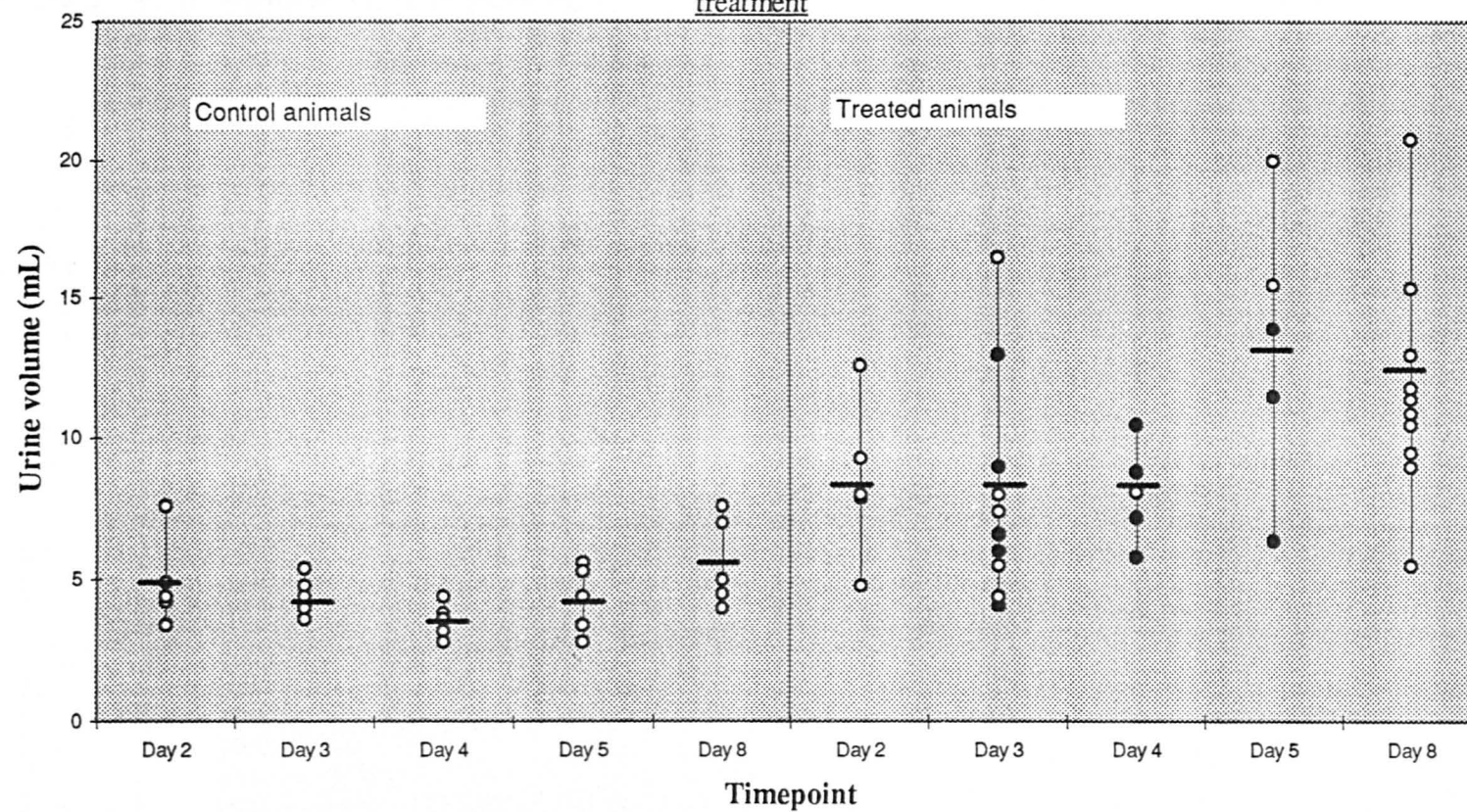
An increase in the number of epithelial cells was seen in treated animals on days 3, 4 and 5 (i.e. after 2, 3 and 4 days' treatment with 500 mg/kg/day ethoxyquin; Table 4.7 and Figure 4.6). There were in excess of 50 such cells per x200 microscope field in those animals with increased numbers (compared to less than 10 in the whole sediment in control animals). This increase was most apparent after 2 days' ethoxyquin treatment (Fig. 4.6). The number of cells was reduced to nearer control levels after 7 days' treatment. The type of epithelial cell involved, and exact cell counts, could not be clearly distinguished due to indistinct morphology in the preparations, possibly caused by technical problems with the staining procedure. Mean urinary volume was increased in treated animals at all timepoints (Table 4.7, Fig. 4.7); this increase was significant at days 5 and 8 where only 1 animal per timepoint was within the relevant control range for urine volume.

Table 4.7: Urine analysis results

Group/ Timepoint		Increased cells in sediment *	Mean urine volume, ml \pm SD
Control:	Day 2	0/5	4.96 \pm 1.63
	Day 3	0/5	3.97 \pm 0.89
	Day 4	0/5	3.58 \pm 0.60
	Day 5	0/5	4.32 \pm 1.20
	Day 8	0/10	5.60 \pm 1.56
Ethoxyquin:	Day 2	0/5	8.54 \pm 2.76
	Day 3	5/10	8.15 \pm 4.17
	Day 4	4/5	8.08 \pm 1.76
	Day 5	3/5	13.50 \pm 4.99
	Day 8	0/10	12.60 \pm 4.10

* Number of affected animals/number of animals in group.

Figure 4.7: Urine volumes and incidence of increased cells in sediment following ethoxyquin treatment



Key: O = Normal number of cells in urine; ● = increased numbers of cells in urine; — = mean

Points on the graph represent individual animals

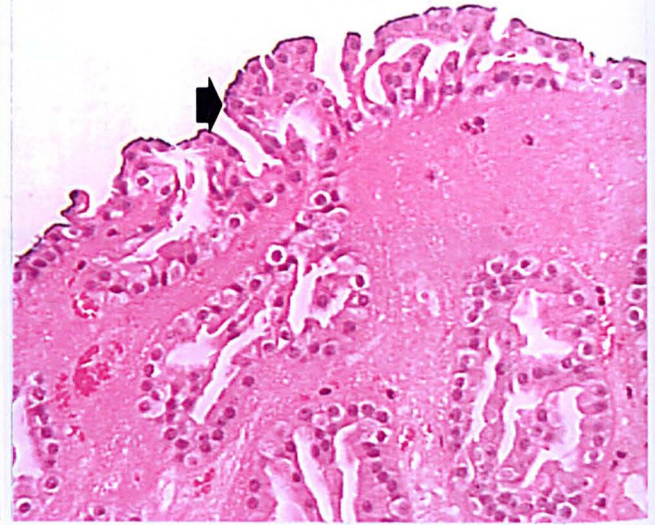
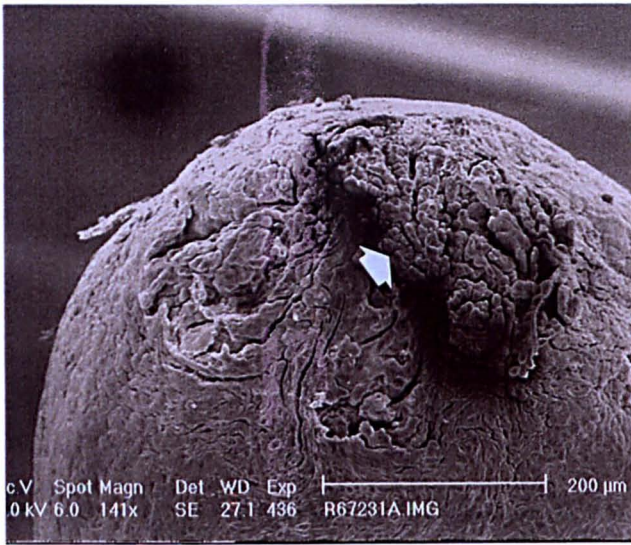


Figure 4.8:

A = SEM photograph of papilla from ethoxyquin-treated animal, showing thickening of surface epithelia (arrow).

B = Subsequent H&E section (x 250) from same papilla, showing hyperplasia and hypertrophy of surface epithelial cells (arrow). Note also moderate RPN in area behind tip.

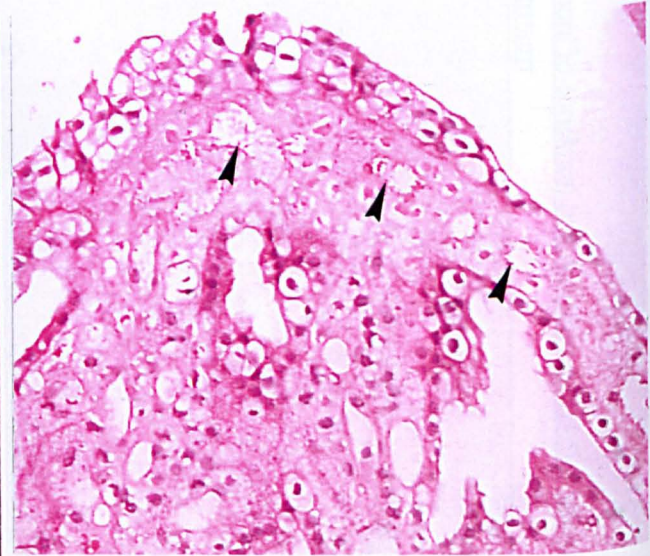
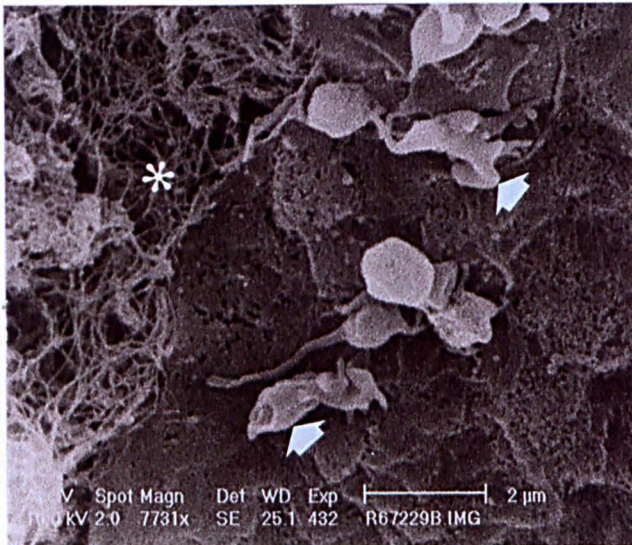


Figure 4.9:

A = SEM photograph of papilla from ethoxyquin-treated animal, showing activated platelets, with cellular extensions, adherent to capillary wall (arrow). Note sub-endothelial collagen fibres (*).

B = Subsequent H&E section (x 250) from same papilla, showing mild RPN. Note small, eosinophilic platelets adherent to walls of capillaries (arrows).

4.3.3: Perfusion methods

All kidneys cleared and perfused well macroscopically. Microscopic evaluation of kidney sections showed perfusion to be incomplete in some animals; some blood cells were present in a few capillaries. Preservation of tissues was generally very good, however, and better than with immerse-fixed material (see 4.3.5).

4.3.4: SEM and correlative histology

The results of combining SEM and subsequent histology are detailed in Table 4.8. SEM highlighted some effects of ethoxyquin which have not previously been reported. The surface epithelium at the papilla tip was often thickened due to hypertrophy and/or hyperplasia of epithelial cells; subsequent histology performed on the same sample showed the surface epithelium to be several cells thick or have much thicker epithelial cells than papillae from control animals (hyperplasia and hypertrophy respectively: Fig 4.8 compares SEM and histology).

SEM also highlighted some capillaries in 2/10 papillae from animals treated for 7 days which contained large numbers of activated platelets with multiple cytoplasmic processes; these were often adherent to capillary walls. Activated platelets were apparent in the H&E sections from the SEM material within capillaries in areas of papillary necrosis (Fig. 4.9 compares SEM and histology). Some "distortion" was noted in the papillae of some treated animals during SEM assessment, this was seen in histological sections as a slight alteration in interstitial structure ("tangled" appearance) without obvious cell loss or necrosis.

Table 4.8: Observations from SEM and Correlative Histology

Observations	Controls		Day 3 ethoxyquin-treated		Day 8 ethoxyquin-treated	
	SEM	Histology	SEM	Histology	SEM	Histology
NAD	10/10	10/10	1/10	1/10	0/10	0/10
Distortion	0/10	0/10	5/10	5/10	0/10	0/10
Thickened epithelium	0/10	0/10	3/10	3/10	10/10	10/10
RPN	0/10	0/10	4/10	4/10	10/10	10/10
Platelets present	0/10	0/10	0/10	0/10	3/10	3/10
Kidney cortex	N/A	NAD	N/A	NAD	N/A	NAD

The numbers in the tables represent the incidence within a group for each finding, where N=10 animals in each group.

KEY: NAD = no abnormalities detected

RPN = renal papillary necrosis

N/A = not applicable

Table 4.9: Summary of TEM observations in ethoxyquin-treated animals

Timepoint	Thickened surface epithelium	Increased platelets present	Incidence of RPN				
			NAD	Degeneration	Minimal	Mild	Moderate
Day 2	5/5	0/5	2/5	3/5	0/5	0/5	0/5
Day 3	5/5	1/5	0/5	4/5	1/5	0/5	0/5
Day 4	5/5	1/5	0/5	3/5	1/5	1/5	0/5
Day 5	5/5	4/5	0/5	2/5	2/5	1/5	0/5
Day 8	5/5	5/5	0/5	0/5	2/5	2/5	1/5

The group size per timepoint was 5 animals, the numbers in the table are incidences of findings.

NAD= no abnormalities detected.

4.3.5: Time-course of lesion development using TEM

Control animals

TEM of control animals showed slight shrinkage of cells with occasional interstitial gaps, but otherwise structures were well preserved (Figures 4.10, 4.11, 4.12). Some dark cells were present among collecting duct cells and surface epithelial cells (these were also seen in some areas of sections from treated animals). Flattened platelets were occasionally present in some capillaries.

Ethoxyquin-treated animals

The findings from ethoxyquin-treated animals over the time-course are summarised in Table 4.9.

Day 2 ethoxyquin-treated animals

Surface epithelial cells were thickened in all animals. Gaps were observed in interstitial areas and vacuolation of some interstitial cells was evident nearer the papilla tip - this was cell "distortion" (derangement) and degeneration (pale outlines) rather than definite necrosis, and was present in 3/5 animals (Figure 4.13). Some dark cells were present among collecting duct cells and surface epithelial cells; these were also seen in control animals.

Day 3 ethoxyquin animals

Gaps and distortion (degeneration) of interstitial areas were evident near the papilla tip in 4/5 animals, as seen in the affected Day 2 animals. This had progressed to focal, minimal necrosis in the remaining animal with definite necrosis of interstitial cells; cells were pyknotic or absent, leaving only cellular debris in the interstitium (Figure 4.14). Possible early necrosis was also evident in some capillary endothelial cells in capillaries (Figure 4.15). Epithelial cells on the papilla surface were thickened or 2-deep in all animals, as noted in the histology observations. Platelets were seen in some capillaries of the animal with RPN, these did not have an activated morphology.



Figure 4.10: Electron micrograph of control papilla, showing well preserved interstitial cells (large arrow) and capillaries (small arrow). x 3500.

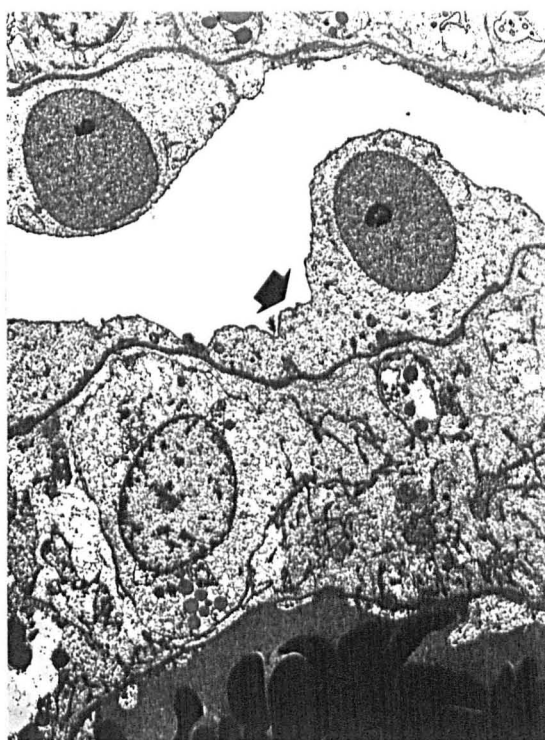


Figure 4.11: Electron micrograph of control papilla, showing normal ultrastructural appearance of loop of Henle (arrowed) in addition to normal interstitium. x 3500.

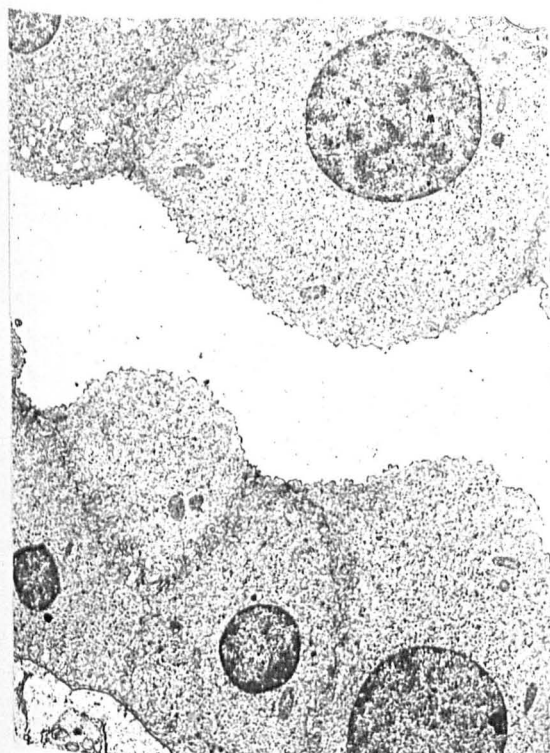


Figure 4.12: Electron micrograph of control papilla showing normal collecting duct epithelium. x 3500.

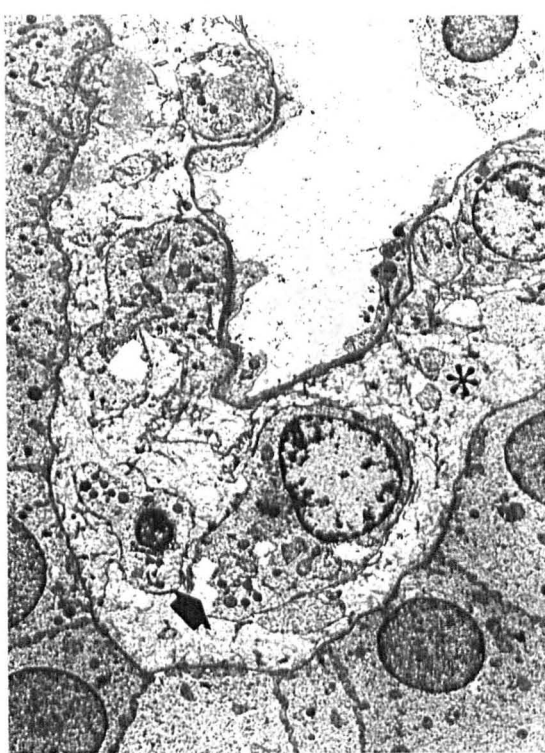


Figure 4.13: Electron micrograph of Day 2 ethoxyquin-treated papilla, showing degeneration of interstitium (arrow) with gaps apparent in interstitial space (*). x 3500.

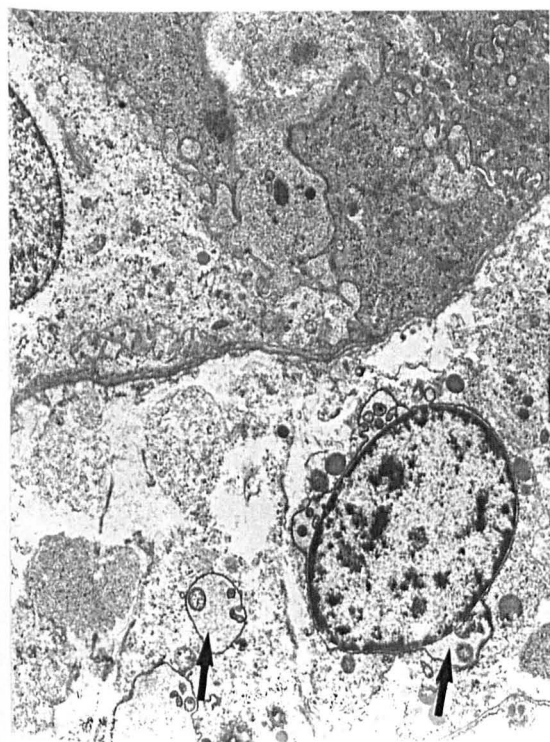


Figure 4.14: Electron micrograph of Day 3 ethoxyquin-treated papilla, showing necrosis of interstitial cells (arrowed) and interstitial debris. x 5850.

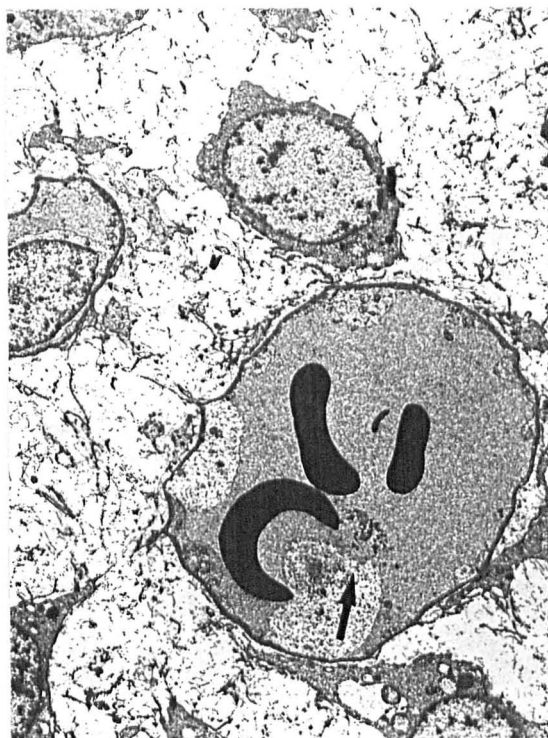


Figure 4.15: Electron micrograph of Day 3 ethoxyquin-treated papilla, showing early damage to capillary endothelial cells (arrow). x 3500.

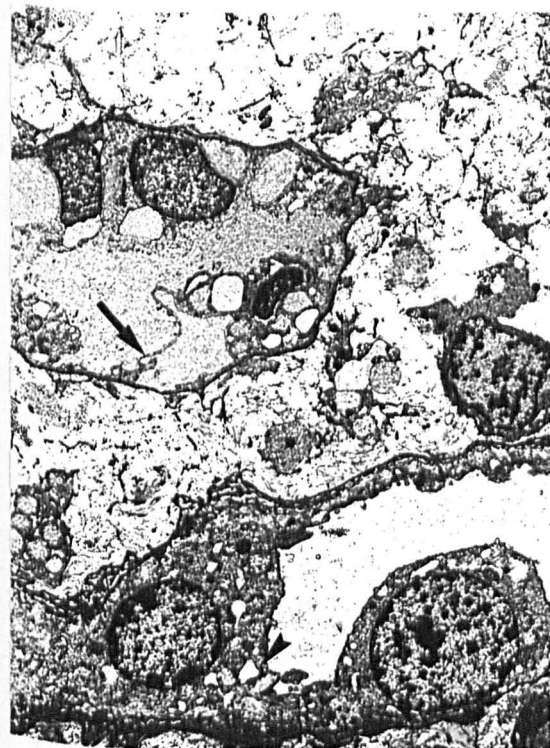


Figure 4.16: Electron micrograph of Day 4 ethoxyquin-treated papilla, showing damage to capillary endothelium (arrow) and loops of Henle (arrowhead). x 3500.



Figure 4.17: Electron micrograph of Day 4 ethoxyquin-treated papilla, showing activated platelets present within damaged capillary (arrow). x 5850.

Day 4 ethoxyquin animals

Surface epithelial cells were thickened or several cells deep in all animals. Gaps and distortion of the interstitia (degeneration) were evident in 3/5 animals; this had progressed to focal RPN in the remaining 2 animals, one with minimal and one with mild RPN. Early damage (loss of some endothelial or tubule epithelial cells) was observed in some capillary walls and loops of Henle, this was more obvious in the animal with mild RPN (Figure 4.16). Activated platelets were also apparent in capillaries within the area of necrosis in the papilla with the highest grade RPN. These platelets had cytoplasmic processes which were often in contact with the endothelial surface, and often had a more rounded appearance than "normal" platelets (Figure 4.17).

Day 5 ethoxyquin animals

Surface epithelial cells were thickened or several cells deep in all animals. Gaps and distortion of the interstitia (degeneration) were apparent in 2/5 animals, this had progressed to RPN in the remaining 3 animals, where only interstitial debris was observed (Figure 4.18). Platelets were observed in some capillaries in those animals with RPN; these were activated in 2 animals with RPN, one of which had the highest grade RPN. Early damage (loss of some endothelial or tubule lining cells) was observed in capillary walls and loops of Henle in the areas of interstitial necrosis, as seen in Day 4 animals. Occasional dark cells were present in some collecting ducts.

Day 8 ethoxyquin animals

By Day 8, thickened surface epithelial cells were apparent in all animals, collecting duct cells also appeared thicker near the tip. Some surface epithelial cells also contained moderate numbers of highly electron dense particles which were bounded by a single membrane and were probably lysosomes. These epithelial cells also had increased intercellular spaces at the cell junctions (Figure 4.19). RPN was present in all animals, this ranged from minimal to moderate in severity (Table 4.9),

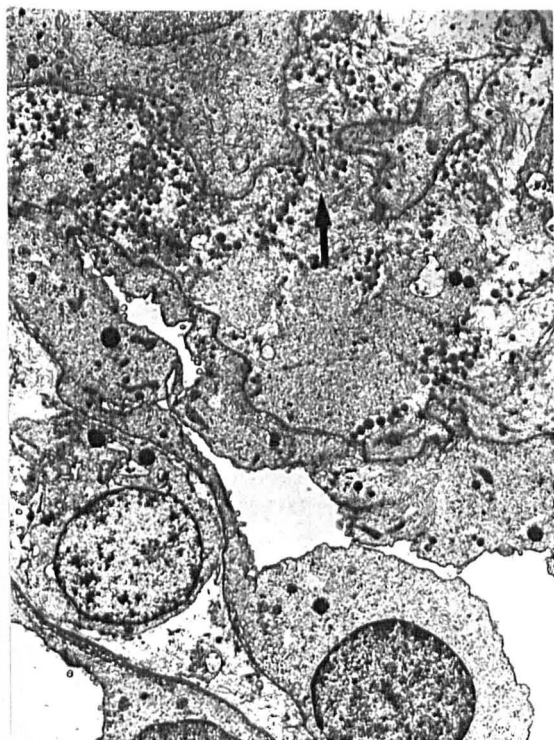


Figure 4.18: Electron micrograph of Day 5 ethoxyquin-treated papilla, showing further necrosis of interstitial cells with fibrillar material within interstitial debris (arrow). x 4425

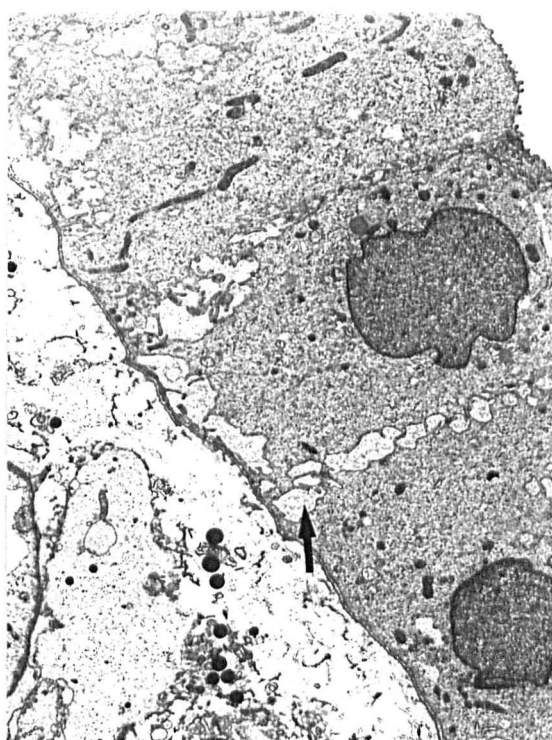


Figure 4.19: Electron micrograph of Day 8 ethoxyquin-treated papilla, showing increased intercellular spaces between surface epithelial cells (arrow). x 4425.



Figure 4.20: Electron micrograph of Day 8 ethoxyquin-treated papilla, showing marked interstitial damage (arrow) and activated platelets present within damaged capillaries, often adherent to endothelium (arrowhead). x 4425.



Figure 4.21: Electron micrograph of Day 8 ethoxyquin-treated papilla, showing more obvious necrosis in loops of Henle, with sloughing of epithelial cells (arrows). Note also platelets within capillaries (arrowhead). x 3500.

where very few interstitial structures were apparent. Increased numbers of platelets were seen in all animals, these were often activated (Figure 4.20). Necrosis of endothelial cells was apparent in some capillary walls and loops of Henle were also affected in areas of necrosis (Figure 4.21). Occasional shrunken/dark collecting duct cells were seen.

Immerse-fixed material

Papillae that were immerse-fixed from animals treated for 2 and 7 days showed similar changes to those seen in the perfused samples, although the preservation of cells and tubules was not as good; an increase in the amount of shrinkage of cells and structures was apparent compared to the perfuse-fixed material from the same timepoints.

4.3.6: Urine Analysis

Levels of conjugated bilirubin were raised in the urine samples from treated animals relative to controls at all timepoints (Table 4.10 and Figure 4.22). No other positive findings were noted: there were no treatment-related changes in glucose, ketones, specific gravity, blood, pH, protein or urobilinogen (Table 4.11).

Table 4.10: Urine clinical chemistry results: Bilirubin changes as indicated by Clinitek analysis

Group/Timepoint	Urine Bilirubin			
	Negative	Small	Moderate	Large
Control: Day 2	4/5 (80%)	1/5 (20%)	0/5	0/5
Day 3	5/5 (100%)	0/5	0/5	0/5
Day 4	2/5 (40%)	3/5 (60%)	0/5	0/5
Day 5	1/5 (20%)	4/5 (80%)	0/5	0/5
Day 8	6/10 (60%)	4/10 (80%)	0/5	0/5
EQ: Day 2	0/5	1/5 (20%)	2/5 (40%)	2/5 (40%)
Day 3	0/10	0/10	4/10 (40%)	6/10 (60%)
Day 4	0/5	1/5 (20%)	3/5 (60%)	1/5 (20%)
Day 5	0/5	2/5 (40%)	0/5	3/5 (60%)
Day 8	0/10	1/10 (10%)	6/10 (60%)	3/10 (30%)

Key: EQ = ethoxyquin

The numbers in the table are number of animals, group size is denoted to the right of the /
The % of animals at each bilirubin level for each timepoint is given in brackets.

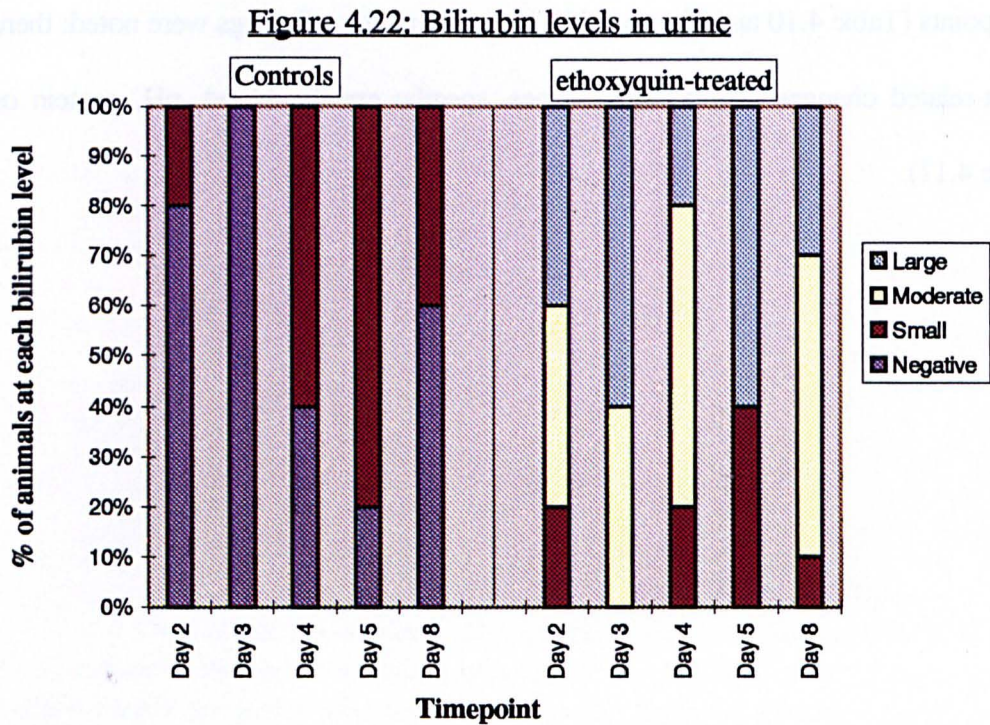


Table 4.11: Urine clinical chemistry parameters unaffected by ethoxyquin treatment

Group/ Timepoint	Glucose (mmol/L)	Ketones (mmol/L)	Specific gravity	Blood present	pH	Protein (g/L)	Urobil- inogen (umol/L)
C / Day 2	-	-	1.02-1.025	-	8.5	0.3	16 (1/5)
Day 3	-	-	1.025	-	7.5-8.5	0.3	3.2
Day 4	-	-	1.025-1.03	-	7.5-8.5	0.3-1.0	3.2
Day 5	-	-	1.02-1.03	-	7.0-8.5	0.3-1.0	16 (1/5)
Day 8	-	-	1.025-1.03	-	8.0-8.5	0.3	3.2
EQ/ Day 2	-	-	1.02-1.03	-	8.0-9.0	0-0.3	16 (1/5)
Day 3	-	-	1.025-1.03	-	7.5-8.0	0.3	3.2
Day 4	-	-	1.02-1.03	-	7.0-8.0	0.3	3.2
Day 5	-	-	1.02-1.03	-	7.0-8.0	0.3	3.2
Day 8	-	-	1.02-1.025	-	7.0-8.0	trace	16 (1/5)

Key: C = Controls; EQ = ethoxyquin-treated; - = negative

The numbers in brackets under urobilinogen are the numbers of animals in the group with this value. All other animals had values of 3.2 umol/L.

4.3.7: Clinical chemistry/Haematology

4.3.7.1: Clinical chemistry

The results from the clinical chemistry assays did not indicate any obvious liver or renal toxicity (Table 4.12). There were slight alterations in some parameters in ethoxyquin-treated animals: decreased albumin, increased total bilirubin and increased sodium at both timepoints; increased lactate dehydrogenase, chloride and globulin and decreased potassium and glucose were observed on Day 3; and increased aspartate aminotransferase on Day 8 (Table 4.12). These changes were mainly due to single animals within the group, with no obvious pattern to these changes to indicate damage to a particular tissue.

Table 4.12: Results of Clinical chemistry analysis of blood following daily dosing with ethoxyquin

Parameter/units	Day 3		Day 8	
	Control	ethoxyquin	Control	ethoxyquin
Albumin g/L	28.88 ±0.64	27.64* ±0.76	29.82 ±0.91	26.56* ±1.08
AST U/L	89.40 ±12.0	111.60 ±25.30	73.20 ±5.40	86.40* ±2.80
LDH U/L	139.40 ±54.3	276.80* ±132.8	132.20 ±90.6	132.80 ±35.1
Total bilirubin umol/L	1.70 ±0.25	2.28* ±0.45	1.34 ±0.19	1.74* ±0.27
Sodium mmol/L	138.95 ±3.42	144.40* ±1.89	143.75 ±0.45	146.53* ±1.74
Potassium mmol/L	6.34 ±0.75	5.20* ±0.42	5.17 ±0.98	5.18 ±0.60
Chloride mmol/L	100.26 ±1.45	104.40* ±1.89	99.44 ±2.11	102.38 ±2.49
Globulin g/L	14.40 ±1.1	16.20* ±0.80	17.60 ±1.50	19.80 ±1.90
Glucose mmol/L	10.64 ±1.33	8.32* ±0.64	9.09 ±1.32	8.85 ±1.62
ALP U/L	428.00 ±90.5	554.00 ±134.5	522.00 ±150.2	376.80 ±83.1
ALT U/L	64.40 ±5.20	68.00 ±20.30	64.60 ±7.50	78.40 ±14.9
Urea mmol/L	3.78 ±0.43	3.82 ±1.33	3.52 ±0.82	3.02 ±1.69
Calcium mmol/L	2.79 ±0.09	2.75 ±0.06	2.75 ±0.12	2.91 ±0.14
Cholesterol mmol/L	2.86 ±0.58	3.22 ±0.44	2.80 ±0.73	3.62 ±0.48
Creatinine umol/L	46.80 ±1.30	45.60 ±2.30	51.60 ±3.60	50.40 ±3.30
Phosphate mmol/L	3.38 ±0.94	2.97 ±0.63	2.59 ±0.95	2.65 ±1.01
Total protein g/L	43.20 ±0.80	43.80 ±1.10	47.60 ±2.20	46.40 ±2.50
Triglycerides mmol/L	0.62 ±0.25	0.93 ±0.23	0.53 ±0.16	0.54 ±0.18

Means ± SD; N= 5 animals per group at each timepoint

Key:

- AST = aspartate aminotransferase
- LDH = lactate dehydrogenase
- ALP = alkaline phosphatase
- ALT = alanine aminotransferase

* = group mean significantly different from control at p < 0.05
** = " " " " " " " " at p < 0.01

Figure 4.23: Haematology findings following ethoxyquin treatment

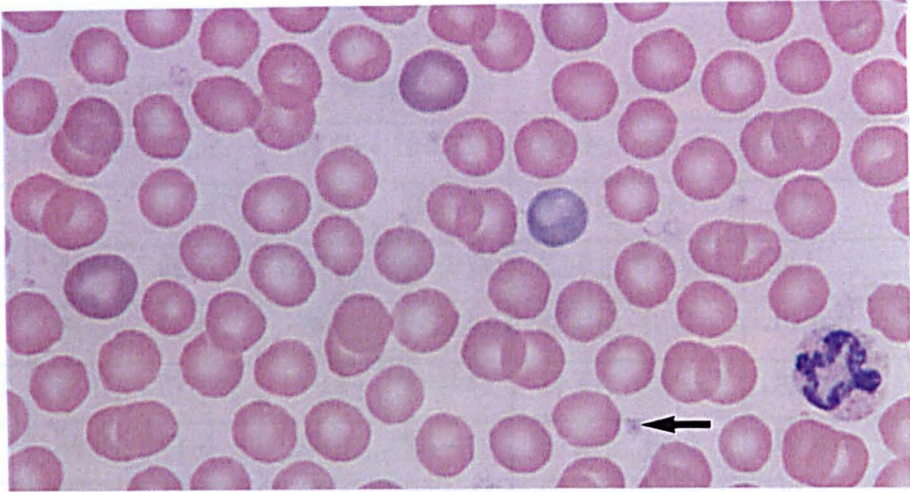


Figure 4.23a: Blood smear from control animal showing normal cell morphology. Note small number of platelets (arrow). Wright's stain, x 1250.

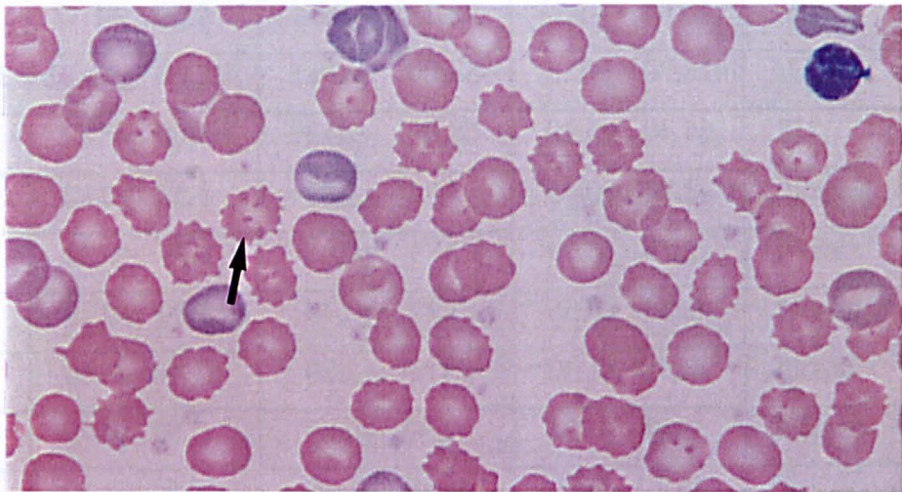


Figure 4.23b: Blood smear from ethoxyquin-treated animal at Day 3. Note crenated appearance of some red blood cells (arrow) and slight increase in platelets. Wright's stain, x 1250.



Figure 4.23c: Blood smear from ethoxyquin-treated animal at Day 8. Note echinocytes ("spikey" red blood cells, arrow), and obvious increase in platelets. Wright's stain, x 1250.

4.3.7.2: Haematology

Animals dosed with ethoxyquin for 2 days (sampled on Day 3) had significantly decreased haemoglobin levels (108 g/L v. 115.8 g/L, $p < 0.01$) and significantly raised numbers of platelets (1291 v. 708.3, $p < 0.05$) compared to controls (Table 4.13). However, the platelet data from this timepoint must be interpreted with caution since some control group animals appeared to have small clots in their blood, thus lowering their platelet count. Red cell morphology was affected by ethoxyquin treatment; cells appeared crenated and in some cases "spikey" (echinocytes) (Figure 4.23).

Animals dosed with ethoxyquin for 7 days had marginally lower haemoglobin levels but these were not significant ($p > 0.05$). Platelet levels were significantly increased over control levels, more so than after 2 days treatment ($p < 0.01$). Again, red blood cells were affected morphologically by ethoxyquin treatment, and much more so than after 2 doses (Fig. 4.20)

Table 4.13: Effects of ethoxyquin treatment on haemoglobin and platelets

Group and timepoint		Haemoglobin level g/L \pm SD	Platelet count $\times 10^9 \pm$ SD
Control	Day 3	115.8 \pm 1.5	708.3 \pm 323.3
ethoxyquin-treated	Day 3	108.0** \pm 3.6	1291.3* \pm 104.5
Control	Day 8	118.0 \pm 8.2	1051.2 \pm 72.2
ethoxyquin-treated	Day 8	115.0 \pm 4.1	1650.4** \pm 169.6

Key:

N = 5 per group at each timepoint

* = group mean significantly different from control at $p < 0.05$

** = group mean significantly different from control at $p < 0.01$

4.3.8: Necropsy & histological assessment of other organs

Macroscopic Observations

The stomachs of some treated animals had areas of white discolouration; in some animals this was in the non-glandular region whereas in others it was noted in the glandular region.

Histological Assessment

The only treatment-related findings seen during histological assessment were in the stomach, where there was mild inflammation with focal ulceration in those stomachs where there had been macroscopic white non-glandular discolouration (as noted to a greater extent in the earlier dose-ranging experiments; 4.3.1.2). No abnormalities were seen in the stomach sections from animals where white glandular discolouration had been noted at necropsy. It is possible that the latter discolouration was due to surface mucus. No effects were seen in the kidney cortex, the liver or any other organ in rats given ethoxyquin.

4.4 DISCUSSION

4.4.1: Development of ethoxyquin as a model for renal papillary necrosis

Dose ranging studies with DMSO

The results of these studies indicated that doses of ethoxyquin up to 800 mg/kg in DMSO (single or 5 daily doses) produced no acute effects in the kidney, with no histological evidence of alteration or necrosis. No difference was seen between male and female rats so males were selected for future experiments as these have been shown to be more susceptible to the renal effects of ethoxyquin [Hard & Neal 1992, Manson et al 1992]. Two strains of male rat were selected for the next stage in the assessment of ethoxyquin (Sprague-Dawley and Fischer rats) to allow inter-strain comparison of any renal effects; Fischer F344 males were used in previous studies which reported RPN following ethoxyquin treatment [Hard & Neal 1992, Manson et al 1992], whilst Sprague-Dawley rats were used for BEA and indomethacin studies in the previous chapter. The next stage in assessment of ethoxyquin was to assess the tolerability and effects of the same concentrations of this compound using a different vehicle (corn oil) in Sprague-Dawley and Fischer rats. The vehicle was changed as DMSO was considered an undesirable vehicle for dosing periods longer than 5 days; peanut oil had been used as a vehicle for ethoxyquin in previously reported studies [Rudra 1974 (vehicle for diet), Skaare & Naftsaad 1979 (oral gavage)] so it was considered that corn oil would be a suitable vehicle to substitute for DMSO.

Change of dosing vehicle and investigation of rat strain for model

The marked lethargy and moribund condition of ethoxyquin-treated animals from Day 2 onwards was totally unexpected and unacceptable; the previous experiment using the same dose concentration, 800 mg/kg, in DMSO for 5 days had produced no clinical signs or histopathology. Furthermore, doses equivalent to up to 1200mg/kg/day, given in the diet for up to 28 days,

produced no adverse clinical signs but did induce RPN [Hard & Neal 1992, Manson et al 1992]. It is possible that the use of corn oil as vehicle markedly increased the bioavailability of ethoxyquin, or perhaps the oil retained the compound in the stomach for a prolonged period, causing the ulceration as a direct irritant effect. This suggestion is supported by Wallig et al [1989] who reported that corn oil delays gastric emptying, and that it can enhance the toxic effects of some compounds.

Withey et al [1983] compared the uptake of pharmacokinetics of lipophilic substances when given by gavage in water or corn oil and showed that the vehicle effects on systemic uptake can be substantial (although they reported that corn oil decreased the rate and extent of uptake of substances when compared to water). Interestingly, Lenz & Carlton [1991] compared the papillotoxicity of diphenylamine (DPA) in male hamsters, when given as an oral dose in DMSO or peanut oil. They also pre-treated hamsters with DMSO before giving DPA in peanut oil to see if DMSO had any protective effects. They found RPN was present in only 1 of 90 hamsters given DPA orally in DMSO and 1 of 30 hamsters pre-treated with DMSO before dosing with DPA in peanut oil. In comparison, DPA given orally in peanut oil induced RPN in 17 of 30 hamsters.

They therefore suggested that DMSO protected against DPA-induced RPN, since no RPN was seen in hamsters compared to dosing in peanut oil by the same route. In contrast to the findings of Lenz & Carlton [1991], Wolf et al [1992a] investigated the protective effects of DMSO against the papillotoxin BEA in Mongolian gerbils, but found that DMSO did not reduce the incidence of RPN. However, in Wolf's study [1992a], DMSO and BEA were injected simultaneously rather than pretreating with DMSO, so the difference in results between the two investigations could be due to this, or there may have been species differences.

The difference in incidence of RPN in Lenz & Carlton's study [1991] could be due to the difference in vehicle, as highlighted by the results of the present experiments where a dose of ethoxyquin that induced no RPN in DMSO proved very toxic and induced RPN in over half the animals when given in corn oil. However, it is possible that DMSO does have a protective effect as proposed by Lenz & Carlton [1991], and that no RPN was seen with DMSO in the present experiments because of this. Lenz & Carlton [1991] reported that DMSO can decrease the vasoactive mechanisms associated with ischaemia as it is a hydroxyl free radical scavenger in vitro and in vivo, and suggested that this may be how it protects against RPN. Ethoxyquin is an antioxidant and also acts as a free radical scavenger, so it is possible that DMSO prevented any papillary effects of ethoxyquin by also scavenging available free radicals and thus reducing the action of ethoxyquin. It would be interesting to further investigate this by pre-treating rats with DMSO, then dosing with ethoxyquin in corn oil to determine whether the incidence of RPN was reduced. The marked difference in the effects of ethoxyquin produced with the different vehicles also highlights a common problem in toxicology, the choice of an appropriate vehicle.

Both strains of rats were affected by RPN although Sprague-Dawley rats were less severely affected by stomach ulceration and renal cortical necrosis, and consequently were less affected clinically. Using this strain would therefore be of benefit in helping to develop a dose regime which would exclude extra-renal toxicity, so Sprague-Dawley rats were chosen for future work with ethoxyquin. The strain differences seen in this experiment demonstrated an issue that can arise in the safety evaluation of a compound when susceptibility differs between strains. However, this strain susceptibility can also be used to advantage when investigating the particular effects of a compound, by selecting the strain that is most susceptible.

Bokelman et al [1971] looked at Charles River Sprague-Dawley (CD) and Manor Farms SPF rats* given NSAIDS and found RPN and haematuria in both control and treated SPF rats, although these findings were more marked in treated animals. They also reported focal hyperplasia of the covering epithelium of the papilla. Examination of Sprague-Dawley control rats showed no RPN or haematuria, but slight interstitial necrosis was seen in the papilla of treated rats, without haematuria. This work demonstrated a problem that can arise in safety evaluation where the presence of strain-specific lesions can make interpretation more difficult.

Of more direct relevance are the findings of Owen & Heywood [1986], who investigated strain-related susceptibility to nephrotoxicity induced by aspirin and phenylbutazone. They compared male and female Fischer F344 and Sprague-Dawley CD rats, those used in the work detailed in this chapter, as well as Wistar and Lister-Hooded rats. They found that although there were no strain differences between Sprague-Dawley and Fischer F344 rats in the appearance of the papillary changes induced by phenylbutazone, the incidence of this necrosis was slightly greater in Sprague-Dawley rats. Furthermore, Fischer F344 rats were more susceptible to the gastrointestinal effects of this compound, necessitating a reduction in dose for this strain. The results reported by Bokelman et al [1971], and particularly those of Owen & Heywood [1986], are similar to the difference in lesion severity seen between Fischer and Sprague-Dawley rats in the work described in this chapter.

The results of the work described in this chapter suggested that ethoxyquin could be a good model for focal necrosis with the potential to be more "reliable" than indomethacin, provided the extra-

* No further information is given in the reference as to the exact strain of this rat.

renal toxicity could be ameliorated. The clinical signs were recognisable at an early stage and the course they would take was known, so that animals could be monitored carefully for the appearance of the early signs. The aim of the next phase of work, a dose-ranging study with reduced concentrations of ethoxyquin in corn oil, was to attempt to induce RPN without stomach ulceration and no cortical damage. It was also hoped to clarify, if possible, whether the effects of ethoxyquin seen with this experiment were due to the change in dose regime (twice daily) or vehicle (corn oil) or a combination of both.

Establishment of optimum dosing regime with corn oil vehicle

The histology findings showed a direct relationship between treatment level, duration of dosing and incidence of RPN. However, the severity of lesions was not obviously dose-related and the extent of the lesion varied between minimal and moderate in all dose groups. It proved possible to induce RPN in all animals from the higher dose groups (500 and 600 mg/kg) after 8 days and in all ethoxyquin-treated animals above a dose of 400 mg/kg after 15 days, without the adverse clinical signs or cortical necrosis seen in the kidney in the previous experiment.

The one death on study was due to acute alveolar haemorrhage and mild inflammation. Mis-dosing via gavage was the most likely cause but it is possible that ethoxyquin could have a systemic effect which affects the lungs. However, this was not seen in any other animal treated with ethoxyquin in any of the experiments conducted, nor has it been reported in the literature.

The highest dose level of ethoxyquin, 600 mg/kg, whilst giving 100% incidence of RPN after 8 days, also caused stomach and renal cortex lesions. The stomach ulceration appeared to be an acute but transient effect of ethoxyquin in corn oil since it was present at day 3 (2 doses), less marked at Day 8 and absent by Day 15. It is apparent that ethoxyquin in corn oil at higher doses has an irritant

effect on the stomach lining. This is supported by the findings of the previous experiment where ethoxyquin in corn oil at 800 mg/kg/day caused severe stomach ulceration after 2-3 days treatment. A dose level of 500 mg/kg ethoxyquin induced RPN in one animal at day 3 and in all animals from Day 8 onwards. No other effects of treatment were seen in the cortex or stomach. An ethoxyquin dose of 400 mg/kg induced RPN in 6 of 8 animals after 8 days and all animals after 15 and 29 days. The lower doses of ethoxyquin only induced RPN in one animal (200 mg/kg) or not at all (100 mg/kg). The results of this time-course study supported the results of the previous experiments which indicated that ethoxyquin could be a good future model for inducing and studying focal RPN. The lesions produced with ethoxyquin appear reproducible and inducible in all animals at the appropriate dose level and dosing period. A dose of 400mg/kg for 15 days or 500mg/kg for 8 days would appear to induce 100% RPN without any other effects; further investigations were aimed at confirming this.

A dose regimen of 500 mg/kg for 8 days was chosen for the subsequent studies with ethoxyquin. The investigations were aimed at evaluating the techniques identified as potentially useful in the previous chapter, SEM and urinary sediments, and investigating whether this dose regime induced any other effects. So far little (or nothing) has been reported about the sequence of events and possible mechanisms of RPN induced with ethoxyquin; TEM evaluation of papillae was included in future work to provide important basic knowledge about this model, including the primary cell type affected and the sequence of events in development of the lesion. Clinical chemistry, haematology, and full necropsy with histological sampling of all organs were included in later experiments with ethoxyquin to determine whether any extra-renal effects of ethoxyquin were apparent or to clarify any findings noted in the course of prior experiments. These investigations were to assess whether ethoxyquin was a site-specific toxin and therefore a suitable model for future use.

4.4.2: Urinary sediments and urine analysis

Preliminary urine analysis in terms of urine appearance, urinary sediment examination and basic clinical chemistry tests (Clinitek analysis) showed some differences between treated and control animals. There was an increase in urinary volume and in brown discolouration of urine in treated animals. Increases in urine volume associated with RPN have been noted with some other papillotoxins, such as BEA [Murray et al 1972, Stonard et al 1987, and the experiments described in Chapter 3], and d-Ormaplatin [Kolaja et al 1994]; the increased urine volume (polyuria) probably occurs due to reduced urine concentrating ability in renal disease, but the mechanism has not been investigated. Reduced urine concentrating ability is one of the features of analgesic and NSAID-induced RPN [Bach & Bridges 1982 & 1985]

The brown discolouration of urine in treated animals may be due to the presence of a metabolite of ethoxyquin. Wilson & DeEds [1959] and Wilson et al [1959] also reported dark urine in ethoxyquin-treated rats, which they said was due to a metabolite of ethoxyquin, although they didn't expand on this. This metabolite could be ethoxyquin nitroxide (EQN) which was proposed as the major metabolite of ethoxyquin by Lin & Olcott [1975]. However, the brown discolouration may reflect increased conjugated bilirubin observed in the urine of treated animals. The liver has been reported as a target organ for ethoxyquin toxicity [Nafstaad & Skaare 1978, Sigma Diagnostics data sheet for ethoxyquin], so it is possible that this increase in bilirubin is due to liver effects (increased bilirubin glucuronide may be present in the urine with hepatocellular damage). Histological sampling of liver and clinical chemistry were therefore included in the subsequent ethoxyquin experiment to determine what other effects of ethoxyquin, if any, were present. However, no obvious liver effects were detected (histological and clinical chemistry findings are discussed in more detail in 4.4.5 and 4.4.6).

Increased numbers of epithelial cells were seen in the urine sediments; this increase in cells was not apparent before Day 3 and peaked at Day 4; fewer animals were affected at Day 5 and by Day 8 numbers were back to control levels. The period over which increased numbers of cells were present correlated well with the time-frame over which ethoxyquin damage was first seen by electron microscopy; most of the changes leading to RPN developed between Days 2 and 5, so it may be expected that more cells would be present in the sediment over this period (The TEM findings are discussed in detail later, in section 4.4.4). The exact cell type involved (transitional or tubular) could not be distinguished due to similarity in morphology between the two and indistinct morphology within the preparations. Whichever type was present, this indicated shedding of cells from either the bladder, the kidney tubules or both. Histological examination of the bladder was included in the subsequent experiment to clarify this. However, no treatment-related effects were seen in the bladder on histological examination (as discussed below) so it seems highly likely that the cells came from the kidney.

4.4.3: SEM and correlative histology

The techniques evaluated in the previous chapter appeared to be useful in identifying the effects of ethoxyquin. SEM proved successful in identifying all animals with either structural distortion or RPN, confirmed by subsequent ("secondary") histology, with no false negatives. Furthermore, it also identified an effect not previously seen with ethoxyquin, that of thickening of the surface epithelium of the papilla. Subsequent histology of the SEM samples further clarified this thickening of the surface epithelium, showing it to be thickened or even 2-3 cells deep in the more affected papillae. SEM has been used for examination of the bladder epithelium of Fischer F344 rats following ethoxyquin treatment [Shibata et al 1991], where ethoxyquin was investigated as a potential bladder cancer promoter. It was found that ethoxyquin caused increased pleomorphism of

the microvilli on the surface epithelium of the renal pelvis, suggesting transient hyperplasia. This is consistent with the observations reported in the papillary epithelium in these experiments, which also suggest hypertrophy and hyperplasia following ethoxyquin treatment.

The combination of SEM and "secondary" histology has proven to be useful in gleaning more information from a single sample, as suggested by the work in the previous chapter with BEA and indomethacin. SEM appears to be a very promising, rapid technique for screening samples for abnormalities, which can be followed by histological examination of the tissues if required. SEM also showed the presence of high numbers of activated platelets in some capillaries in the papillae from 2 treated animals at Day 8, which was further borne out by the TEM examinations as detailed below. The use of SEM for the assessment of papillary damage with RPN has not previously been reported, although SEM has been used to look at sloughing of the papillary epithelium following bacterial infection associated with RPN [Cohen et al 1979, 1981], and to investigate bladder epithelial changes with RPN [Okamura et al 1992]. The use of SEM and correlative histology for the investigation of RPN with any compound, or for the investigation of any other tissues, has not previously been reported in the literature.

4.4.4: Time-course of lesion development using TEM

The TEM evaluation gave important information about the time-course of lesion development with ethoxyquin and the cell types affected. It also highlighted some previously unseen changes as noted in the SEM assessment, namely the surface epithelial thickening and presence of increased numbers of activated platelets. The results suggest that the sequence of events in the development of RPN starts with interstitial "distortion" and some surface epithelial thickening, apparent at Day 2 (24 hours after treatment). By Days 4 and 5 this progresses to RPN in around half of the treated animals with early damage to some capillary walls and then loops of Henle. At this stage activated

platelets are also seen in those animals with RPN. By Day 8, RPN is seen in all animals, with corresponding increases in the numbers of activated platelets present. Haematology investigations were carried out in the final ethoxyquin experiments to investigate the effect of ethoxyquin on erythrocytes and platelets with sampling points at Day 3 and Day 8; the results supported this observation, showing there to be a possible increase in numbers of platelets in the blood at Day 3, and a definite increase at Day 8 (haematology results are discussed further below).

The cause of the surface epithelial thickening (hypertrophy and hyperplasia) and platelet activation seen with both TEM and SEM is unknown, although there are various possibilities. Ethoxyquin or its metabolites may have some effect on growth factors or prostaglandins (for example, PGE₂ is the major prostaglandin produced in the papilla and inhibits platelet aggregation, but its production is inhibited by NSAIDS such as indomethacin and naproxen [Zusman & Keiser 1977]) which could then induce hypertrophy and hyperplasia in surface epithelial cells and cause platelet activation [DeGroot & Sixma 1990]. Alternatively, these may be independent events. The hypertrophy and hyperplasia may be due to a direct action of ethoxyquin or its metabolites on the epithelial cells as they are excreted from the papilla. Subtle damage may occur in capillary walls during development of RPN with ethoxyquin, causing exposure of underlying cellular material (eg collagen) which induces platelet activation [DeGroot & Sixma 1990]. The results of the early time-points from this study suggest that the presence of increased platelets occurs after the surface epithelial thickening and interstitial cell damage and may be secondary to early damage to the capillary endothelial cells.

Increased numbers of activated platelets have been reported with other compounds used to induce experimental RPN, including analgesics. Levin & Abrahams [1968] used TEM to show platelets adherent to the intima of capillaries in rats fed aspirin by gavage, and Burrell et al [1991a] also reported increased platelets in the damaged capillaries of rats treated with aspirin. Gregg & Bach

[1990] demonstrated platelets adherent to the capillary endothelium in nude mice treated with BEA, and Wolf et al [1992a] also showed platelet aggregates with degenerate endothelium in gerbils treated with BEA.

It has been suggested that the presence of increased number of platelets adherent to the endothelia is a precursor to thrombi formation, thus providing evidence to support the theory that ischaemic necrosis is the cause of RPN. However, the results of the present experiments with ethoxyquin and those with BEA which showed platelets adherent to degenerate endothelia [Burrell et al 1991, Wolf 1992a] would suggest that the presence of increased platelets (which may lead to thrombosis) is secondary to damage to the endothelia and necrosis of interstitial cells. It could not be determined whether thrombi were present in the present experiments because of the fixation technique used (perfusion with heparinised saline prior to fixative may have dislodged any small thrombi).

Hyperplasia of the surface epithelium of the papilla associated with RPN has previously been reported in rats following treatment with the analgesics phenacetin [Johansson & Angervall 1976] and aspirin [Molland 1976], and with other papillotoxins, such as dimethylarsinic acid [Murai et al 1993]. Johansson & Angervall reported that vascular changes (thrombus formation) and epithelial hyperplasia were often simultaneously present following phenacetin treatment, but proposed that these were independent events. They said that although the exact mechanism for the hyperplasia reported was unknown, it could be a direct action of metabolites of the compound.

4.4.5: Haematology & Clinical chemistry of blood

The haematology and cell morphology investigations which were included to further investigate the platelet effects seen with SEM and TEM gave some interesting results. The increased number of platelets seen in the blood samples from ethoxyquin-treated animals was consistent with the

increased numbers of platelets seen in capillaries of the papilla with SEM and TEM. The slight decrease in haemoglobin may have been due to haemolysis, although the red blood cell changes (crenation and acanthocytosis) were not accompanied by increased polychromasia (juvenile red blood cells). A more sensitive indicator of red blood cell regeneration would have been to measure reticulocytes; however, an accurate assay for reticulocytes was not available at the time of this experiment.

It is feasible that ethoxyquin exerts an effect on the red cell membrane that is sufficient to cause haemolysis in a small cohort of more sensitive (older) red blood cells. For the remaining population, it may be that ethoxyquin induces a mild effect on red cell membrane integrity which is not sufficient to cause haemolysis in vivo, but induces the morphological effects seen when a blood film is made (ex-vivo). This suggestion is supported by work which reported that ethoxyquin at a dosage of 500 mg/kg (the same dose as used in the present studies) had a marked effect on red blood cell membrane integrity, although it did not produce lysis in vivo [Kumar et al 1979].

Kosaka & Yoshida [1973] also found that ethoxyquin caused increased haemolysis in weanling rats (the age of the rats in these studies) and in chick red blood cells, and Hernandez et al [1993] reported haemolysis in marmosets given an accidental overdose of ethoxyquin in the diet. Furthermore, Kagan et al [1986] examined the effects of various natural and synthetic antioxidants which are widely used as stabilisers of biomembranes against lipid peroxidation. They found that synthetic antioxidants devoid of hydrocarbon tails exerted toxic effects on red blood cell membranes, inducing haemolysis, and caused changes in endoplasmic reticulum and platelet membranes (also initiating calcium-dependent platelet aggregation), whereas antioxidants with long hydrocarbon tails did not induce these effects. Ethoxyquin has only a short hydrocarbon tail, so it is possible that it could induce such effects to a lesser degree, especially if it is oxidised to its more

potent metabolite, ethoxyquin nitroxide [Lin & Olcott 1975].

Jayanthi & Varalakshmi [1992] looked at alterations in red blood cell membranes with a potent antioxidant (DL α -lipoic acid) with regard to its protective effects against calcium oxalate lithiasis. They found that it caused a decrease in the structural changes normally seen with lithiasis as a result of decreased lipid peroxidation. They did not investigate whether the antioxidant alone, in the absence of the countering effects of calcium oxalate lithiasis, would cause any membrane effects because of this decreased peroxidation. It seems likely that this would be the case, given the findings with ethoxyquin and those reported with other antioxidants [Kagan et al 1986].

The clinical chemistry findings showed slight changes in several parameters (increased AST & LDH and decreased albumin), although these did not give an obvious characteristic pattern for any one organ being affected. Red blood cells are a source of LDH and AST (but not ALT), therefore increases in LDH and AST alone may reflect mild haemolysis. Haemoglobinuria was not evident but since plasma haptoglobin must be saturated before haemoglobin is lost via the urine, haemoglobinuria is not a very sensitive marker and its absence does not exclude the presence of in-vivo haemolysis. These changes could therefore be further indication of a minor effect of ethoxyquin on red blood cells.

Increased AST & LDH and decreased albumin could also indicate that there may be some effects in the liver, although these changes were small with no alteration in ALT and no histological changes. Hepatic effects have previously been reported with ethoxyquin [Nafstaad & Skaare 1978] so the changes seen in AST and LDH in the present experiments may indicate minor hepatic damage. If there is an effect of ethoxyquin treatment on the liver, it is subtle and not sufficient at this dose to induce any obvious necrosis (discussed further in 4.4.6). It is also possible that the decreased

albumin levels, raised sodium and chloride and decreased potassium could be related to the renal lesions caused by ethoxyquin.

4.4.6: Full necropsy and histological assessment

The indication that ethoxyquin did not cause any obvious liver effects at the dose used to induce RPN is important for the use of ethoxyquin as a model for RPN, since any additional toxicity would affect its potential as a model. The liver has been reported as a target organ for ethoxyquin [Sigma Diagnostics data sheet for ethoxyquin, Nafstaad & Skaare 1978], so it may be expected that it would induce some liver changes, although these may have been too subtle to be detected by light microscopy. Nafstaad & Skaare [1978] reported no histological liver effects following a single dose of 500 mg/kg (the dose concentration used in these experiments), but did detect slight liver changes using electron microscopy. The effects reported were proliferation of smooth endoplasmic reticulum (SER), dilated cisternae of endoplasmic reticulum, and disorganised mitochondrial membranes (the latter could be the origin of the increased AST noted in the present experiments). Kahl [1984], looking at the biochemical action of ethoxyquin, also found increased ER in the liver and increased enzyme induction. He also reported inhibition of lipid peroxidation in biological membranes with ethoxyquin treatment. The reported hepatic effects of ethoxyquin may therefore be related to the membrane effects noted with several antioxidants, including ethoxyquin [Kumar et al 1979, Kahl 1984, Kagan et al 1986]. These membrane effects may also be responsible for the red blood cell findings in the present experiments.

No microscopical effects were seen in any other tissues except for minimal inflammation and focal ulceration in the non-glandular portion of some stomachs. This finding had been noted in the preceding experiments performed with ethoxyquin in corn oil and was shown to be a transient, probably irritant effect of ethoxyquin in corn oil on the stomach. No histological abnormalities were

seen in the glandular area of the stomach where white discolouration had been noted at necropsy. It is possible that this discolouration of the glandular area was due to the presence of surface mucus.

4.4.7: Comparison of findings with previous ethoxyquin work

The first reported work regarding development of RPN following ethoxyquin treatment [Hard & Neal 1990 & 1992] found that RPN was first observed in male F344 rats after 4 weeks of treatment with ethoxyquin, given as 0.5% in the diet. Interstitial degeneration in 3 of 8 males was noted at 4 weeks, which had progressed to RPN after 14 weeks, with complete necrosis and pyelonephritis after 24 weeks. They also reported capillary changes following ethoxyquin treatment. Manson et al [1992], in studies which highlighted the age- and sex-dependent nature of RPN induced with ethoxyquin, also reported RPN in male weanling rats following 20 weeks of treatment, with increased proteinuria.

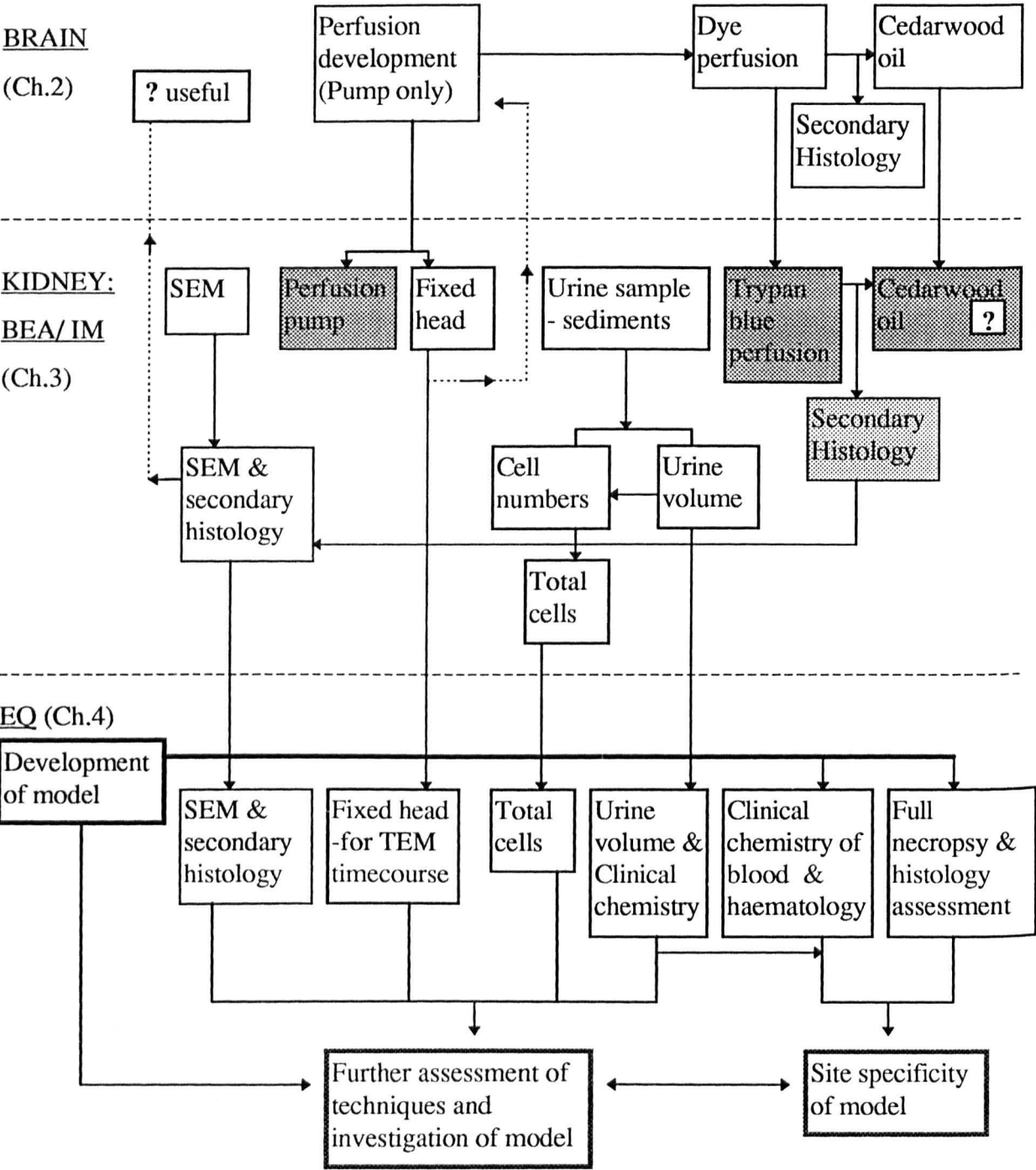
The dose regimen developed in the current experiments (500 mg/kg/day) resulted in reproducible RPN in all male rats by 8 days of treatment, with interstitial degeneration first appearing at Day 2 (after 1 dose of 500 mg/kg). The broad sequence of events reported in the study by Hard & Neal matches that seen in the present experiments, but in much less detail, and over a much longer time-frame. The study by Manson et al [1992] showed the same severity of RPN as Hard & Neal's study after 20 weeks [1992], but the proteinuria noted by Manson et al [1992] was not seen in these experiments. The average daily dose for the rats in Hard & Neal's studies [1990, 1992] (and Manson & co-workers [1992]) would equate to between 1000 and 1200 mg/kg/day, which is twice that used in these experiments. However, ethoxyquin was given "diluted" as part of the diet mix, which would be eaten gradually over several hours, rather than as a single direct dose by oral gavage as was the case in these experiments. Any toxic effects may therefore be expected to be reduced and delayed in comparison, although no pharmacokinetic profiling with ethoxyquin has

been reported by anyone to confirm this hypothesis.

It has therefore proved possible to develop a dose regime using ethoxyquin which reliably and reproducibly induces RPN over a relatively short period. Dosing by oral gavage rather than in the diet meant that the amount of ethoxyquin given could be more easily controlled and a more accurate assessment of the dose-ranging effects with ethoxyquin could be made. Also, a detailed investigation of the sequence of events involved in the development of RPN with ethoxyquin was easier to perform, and comparisons with the effects of other models, given over short periods of treatment, could then be made more easily.

The results of the experiments carried out with ethoxyquin suggest that, using the dose regime developed, ethoxyquin is a site-specific toxin for the kidney with no obvious extra-renal pathology and minimal effects on red blood cells. It therefore has strong potential as a model for studying RPN. If longer periods of dosing were required, for example to assess ethoxyquin as a model for sub-chronic/chronic RPN, this could be achieved by reducing the dose of ethoxyquin, as demonstrated by the results of the dose-ranging experiments where 400 mg/kg induced RPN in all rats and 200 mg/kg induced RPN in 1 of 4 rats after 14 days of treatment. Ethoxyquin therefore also has potential as a more "flexible" model for studying RPN than other current models such as BEA, which is used as a single dose to induce RPN and causes secondary cortical damage and infection after prolonged periods, and indomethacin, which induces marked extra-renal toxicity.

Figure 5.1: Schematic flow diagram of the experimental work carried out during the project



CHAPTER 5. DISCUSSION AND RECOMMENDATIONS

The experimental work carried out during the project is summarised in the schematic flow diagram in Figure 5.1; this work can be divided into two main phases:

1. Development and assessment of techniques to help identify and study focal lesions. Perfusion fixation and dye perfusion techniques were initially evaluated in the brain. Two well-established models of RPN were then used to develop these and other techniques in the kidney. Those techniques which showed promise were then used to investigate a new model of RPN.
2. Development of a new model for RPN. The techniques identified in the first phase of the work were used to help investigate the utility of the antioxidant ethoxyquin as a model for RPN. Other investigations (TEM, clinical chemistry, haematology, full necropsy) were also included at this stage to study the pathological changes induced with this model and determine its potential.

As a consequence of this work, comparisons could then be made between the changes induced with, and possible mechanisms of, the three experimental models of RPN and those seen in man.

5.1: DEVELOPMENT AND ASSESSMENT OF TECHNIQUES

5.1.1: Perfusion fixation

The work carried out in the brain showed that perfusion fixation could successfully be used in toxicology studies for light and electron microscopic examination of brain tissue. After optimising perfusion rates for evaluation of brain tissue using perfusion pump apparatus, the technique was applied to the kidney. Perfusion pump (local perfusion) and fixed head of pressure (whole body perfusion) techniques were evaluated and both methods gave successful results, although the fixed

head of pressure method was more rapid and straightforward. However, local perfusion is recommended where kidneys need to be treated differently to allow greater control (e.g. one kidney left unfixed as a negative control), or where perfusion of the kidneys (or brain and upper body) only is required and other tissues must be unfixed for other toxicological investigations (e.g. biochemical analysis).

Whole body perfusion is recommended for rapid perfusion with minimal intervention; this is particularly suitable for transmission electron microscopy investigations, and was used for the ultrastructural investigations with ethoxyquin to investigate the time course of lesion development with this model of RPN. The whole body perfusion technique (fixed head of pressure) developed for these experiments has also subsequently been applied with success to the brain, and used in a recent toxicology study in the hamster enabling the ultrastructural details of a specific effect in the brain to be studied (unpublished observation).

5.1.2: Dye perfusions

Dye perfusion of the brain gave promising results for examination of the vasculature in the brain work, especially when combined with "clearing" slices of tissue in cedarwood oil and subsequent histology. One of the great advantages of perfusing with dye and fixative is that it allows histological material to be processed from tissue previously prepared to display the vasculature, with very high quality results. When dye is perfused in conjunction with fixative, electron microscopy can also be performed. This technique could be used in future mechanistic studies where ischaemic hippocampal lesions occur, or in other tissues where the vasculature is implicated in the occurrence of a lesion. Using this method it should be possible to visualise alterations and identify the vessels involved, and to identify whether the vasculature is primarily involved in the development of a lesion.

The dye perfusion technique used with the brain was developed and further assessed in the kidney work. The technique of dye perfusion, and subsequent histology, was modified slightly by using a different dye (trypan blue) and assessing various routes of perfusion to evaluate this as a means of identifying necrotic cells in the kidney rather than highlighting alterations in the vasculature. However, perfusion of the kidney with trypan blue was of little success; it did not highlight necrotic areas, and although trypan blue had been successfully perfused into the kidneys, it was retained in some healthy cells rather than excluded by them. The results of the kidney experiments using BEA and indomethacin indicated that trypan blue was too unreliable for use in routine studies, and trypan blue was not investigated further for use in the identification of necrotic renal lesions. Cedarwood oil was also used for the examination of cleared slices of kidney, prior to histological assessment, to assess its use in aiding visualisation of vessels and damaged tubules, but this was not always useful due to the thickness of slices required for subsequent histology.

5.1.3: Assessment of standard histology technique for identifying RPN

The suitability of the standard technique used for identification of RPN, a single section through the kidney, was assessed prior to evaluating techniques which might help in this identification. This initial work reinforced the importance of standardising sectioning for assessment of necrosis [Bach & Bridges 1982 & 1985]. Step-serial sectioning of BEA-treated kidneys, carried out to determine how much the distance from the papilla tip affected the extent of necrosis, showed that damage was most extensive in the papilla tip region, supporting the general assumption that papillary necrosis induced with BEA is most extensive at the tip [Hill et al 1972, Duggin 1980, Bach & Bridges 1982 & 1984]. Furthermore, the standard technique used to assess the extent of damage, a histological section near the papilla tip, was shown to be robust, since the section did not have to be exactly

through the tip, just within 60 μm , to give a reliable estimate of the extent of damage. The histology findings from the kidneys of indomethacin-treated animals highlighted the relatively high number of false negatives obtained when relying on a single histological section through the tip with a focal model. This information was important in highlighting both the limitations of relying on a single section for studies involving focal necrosis and reinforced the need for more reliable techniques in identifying such damage [Arnold et al 1974, Bach & Bridges 1982 & 1985, Price et al 1996].

5.1.4: Urinary sediments

Urinary sediments gave promising results in helping to identify RPN with BEA, indomethacin and, subsequently, ethoxyquin-treated animals when the total number of transitional cells excreted in the urine was calculated (correcting for urine volume gives a more reliable estimate of any effects in the urine, as stressed by Fent et al [1988] and Zbinden et al [1988]). The results generally showed that more cells were present in the sediments of animals with RPN and indicated that urine cytology might be useful to help identify RPN, supporting the findings of Jackson et al [1978], who found sediments useful in screening for carcinoma and dysplasia in analgesic abusers and suggested that urinary sediments might also be useful to help detect RPN in patients.

However, it has been emphasised that cell excretion following kidney damage can fluctuate or disappear with continued treatment [Davies et al 1968, Kennedy & Gail Saliya 1970, Fent et al 1988, Zbinden et al 1988]. The work carried out in this project, particularly the time course studies with ethoxyquin, supported this. Stonard et al [1987] recommended frequent collection of urine samples to enable the onset of, and recovery from, renal lesions to be followed so that transient effects are not overlooked. Similarly, Fent and Zbinden and co-workers [1988] recommended a screening program based on repeated urinalysis as a useful procedure to detect nephrotoxic chemicals acting by a variety of mechanisms. Such a procedure would hopefully highlight those

individuals (animals or patients) where necrosis was present at an early stage in the development of the lesion. In toxicity studies, combining this with histopathology, would provide the maximum information on the renal effects of a compound; whereas in patients, treatment with the offending compound could be stopped in the early stages of lesion development when the chances of recovery are greatest.

5.1.5: SEM and correlative histology

Scanning electron microscopy (SEM) gave promising results with all three models, particularly when combined with secondary histology (reprocessing of SEM tissue for histology) thus providing more information from a single sample. All kidneys identified as having RPN or degeneration by SEM were confirmed as such by secondary histology of the samples. Perhaps the most important observation is that SEM did not produce any false negatives, even with very small lesions. SEM also highlighted important effects on the surface epithelium following BEA and ethoxyquin treatment, which in the case of ethoxyquin had not previously been reported.

SEM appears to be a promising, rapid technique for screening samples for abnormalities, which can be followed by histological examination of the tissues if required. The use of SEM for the assessment of papillary damage with RPN has not previously been reported; the use of SEM and correlative histology for the investigation of RPN with any compound, or for the investigation of any other tissues, has not previously been reported in the literature. This technique also has potential for other tissues; SEM (and subsequent histology) can be performed on standard study material (i.e. tissue immerse-fixed in formalin) with no loss of detail, which makes this technique applicable to any routine studies where RPN or any other type of necrosis may be suspected. SEM may also have potential for investigating focal lesions in the brain following ischaemia; perfusion with latex instead

of dye, and preparation of corrosion casts could be used to study any effects on vasculature [Coyle 1976, 1978] as a complement to the techniques developed here.

5.2: ETHOXYQUIN AS A MODEL FOR RPN

The experimental work carried out to investigate ethoxyquin and assess its potential as a model for RPN raised some very interesting issues, which contribute to what is currently known about this compound in both its reported effects on the renal papilla [Hard & Neal 1990 & 1992, Manson et al 1992] and its proposed effects on other tissues or structures such as the liver and erythrocytes [Kosaka et al 1973, Nafstaad & Skaare 1978, Kumar et al 1979, Hernandez et al 1993]. The differences observed in species susceptibility and subsequent extra-renal effects in the preliminary dose-ranging studies with ethoxyquin (Fischer F344 and Sprague-Dawley rats) support previous work reporting differences in species sensitivity to RPN [Owen & Heywood 1986, Bokelman et al 1986, Lenz & Carlton 1991]. The differences in species sensitivity highlight the importance of selecting the appropriate species for toxicity studies with a compound, and the marked increases seen in clinical signs and lesion incidence following a change in vehicle (from DMSO to corn oil) emphasise the importance of selecting the appropriate vehicle.

A dose regimen was successfully developed with ethoxyquin which reliably and reproducibly induced RPN in male rats over a relatively short period. A dose level of 500 mg/kg/day resulted in reproducible RPN in all male rats by 8 days of treatment, with interstitial degeneration first appearing at Day 2 (after 1 dose of 500 mg/kg). The results of the experiments carried out with ethoxyquin suggest that, using the dose regimen developed, ethoxyquin is a site-specific toxin for the kidney with no obvious extra-renal pathology and minimal effects on red blood cells. It therefore has strong potential as a model for studying RPN. The broad sequence of events reported in

previous studies with ethoxyquin [Hard & Neal 1990 & 1992, Manson et al 1992] matches that seen in the current experimental work, but in much less detail and over a much longer time-frame. Dosing by oral gavage rather than in the diet, which had been used by others in investigations of RPN with ethoxyquin [Hard & Neal 1990, 1992, Manson 1992] meant that the amount of ethoxyquin given could be more easily controlled and a more accurate assessment of the dose-ranging effects with ethoxyquin could be made. Also, a detailed investigation of the sequence of events involved in the development of RPN with ethoxyquin was easier to perform, and comparisons with the effects of other models, given over short periods of treatment, could be made more easily.

If longer periods of dosing were required, for example to assess ethoxyquin as a model for sub-chronic/chronic RPN (as in analgesic-induced RPN in man), this could probably be achieved by reducing the dose of ethoxyquin as demonstrated by the results of the dose-ranging experiments. It would be interesting to further investigate ethoxyquin as a chronic model for RPN, to enable the development of chronic RPN and long term effects of treatment to be studied and related to the effects seen in man. Ethoxyquin therefore also has potential as a more "flexible" model for studying RPN than other current models such as BEA, which is used as a single dose to induce RPN but causes secondary cortical damage and increases the incidence of infection after prolonged periods, and indomethacin, which is also used as a single dose but induces marked extra-renal toxicity.

Table 5.1: Comparison of the pathological changes found with the various models of experimentally-induced RPN and those found in man, based on results of experiments and reported findings

Morphological or functional changes	Type of renal papillary necrosis			
	BEA-induced (single dose)	Indomethacin-induced (single dose)	Ethoxyquin-induced (multiple doses)	analgesic-associated RPN in man (multiple doses)
Necrosis of the papillary tip	yes	yes	yes	yes
Loss of interstitial cells	yes	yes	yes	yes
Loss of vasa recta/ capillaries	yes	yes	yes	yes
Loss of thin loop of Henle	yes	yes	yes	yes
Platelets adherent to damaged endothelium	yes	yes	yes	yes
Hyperplasia of covering epithelia	yes	yes	yes	yes
Loss of collecting ducts and covering epithelia	yes	yes	? at later stages	yes
Lipid changes (e.g. increased in epithelial cells)	yes	yes	yes	yes
secondary cortical changes	yes	yes	yes at higher doses	yes
Urine cytology changes associated with RPN	yes	possibly	yes	possibly
Decreased urine concentrating ability	yes	possibly	yes	yes
Extra-renal pathology	no	yes	yes at high doses	yes
Postulated mechanisms for lesion	Renal concentration & activation, ? vasoconstriction	Metabolic activation, + PG inhibition	Metabolic activation, + PG inhibition	Metabolic activation, + PG inhibition: ischaemia?

5.3: COMPARISON OF MODELS AND PROPOSED MECHANISMS

5.3.1: Pathological changes

The development of RPN induced with ethoxyquin appears to follow the same course as that seen in the two well-established models of RPN, BEA and indomethacin, and agrees with the sequence of events reported in man [Arnold et al 1974, Molland 1978, Kincaid-Smith 1979, Bach & Bridges 1982 & 1985, Bach & Hardy 1985, Gregg et al 1989c, Burrell et al 1990]. Table 5.1 summarises the morphological and functional effects of the various models, using the results of the experiments carried out during this project and those reported in the literature. It is generally agreed that the renal medullary interstitial cells are the first structures affected with RPN, followed by degeneration and necrosis of the endothelial cells of capillaries/vasa recta and the thin loops of Henle. The surface epithelia and collecting ducts of the papilla are spared initially but become affected as the lesion progresses. The lesion may then progress to affect the rest of the papilla and eventually the cortex.

The extensive papillary effects seen in the later stages with BEA and analgesic nephropathy were not seen with ethoxyquin, nor was there loss of the surface epithelium as is seen with BEA and analgesics [Bach & Bridges 1982 & 1985, Bach & Hardy 1985, Gregg et al 1989c]. However, the dose regimen for ethoxyquin was designed to investigate the early development of the lesion rather than its progression to end stage necrosis, so it may be that with continued dosing these changes would occur. Hypertrophy of the surface epithelial cells was evident with ethoxyquin. Such hypertrophy has also been reported prior to loss of the surface epithelial cells following treatment with BEA and analgesics [Molland 1976, Bach et al 1983, Elliot 1986, Gregg et al 1989a & c, 1990a & b, Gregg & Bach 1990, Bach 1991], so it seems reasonable to expect that loss of epithelial cells would also occur with ethoxyquin as the lesion progressed.

Ethoxyquin, in contrast to the other experimental models used, induces RPN following multiple dosing, with RPN gradually developing over the dosing period. The other two models, BEA and indomethacin, cannot be given for longer periods as they induce acute RPN and also cause marked cortical effects and secondary infection (BEA) or marked gastrointestinal effects (indomethacin) in animals after 24-48 hours. Ethoxyquin therefore has more in common with analgesic nephropathy in man in this respect, which also develops as a consequence of multiple dosing and is usually sub-chronic/ chronic in its onset.

5.3.2: Proposed mechanisms for RPN

The currently proposed mechanisms for development of RPN with the different compounds are summarised in Table 5.1. The most common model used for studying RPN, BEA, is generally considered to have a different mechanistic pathway than that proposed for analgesics and NSAIDS [Hill et al 1972, Solez et al 1974, Bach et al 1980 & 1986, Davies & Tange 1982, Bach & Bridges 1985, Powell et al 1985 & 1991, Wilks et al 1986, Thieleman et al 1990, Wolf et al 1990, Stewart 1995]. The proposed mechanistic pathways for the different compounds are illustrated in Figure 5.2.

Development of RPN with BEA is believed to occur as a consequence of accumulation of BEA in the papilla (particularly the interstitial cells) via the counter-current concentration mechanism. This accumulation promotes cyclisation of BEA into potent alkylating agents (reactive intermediates) which induce lipid peroxidation, initiating a cascade of events leading to cellular damage and necrosis (Fig. 5.2). Vasoconstriction has also been proposed to play an important role in BEA-induced RPN by some investigators, although this has been disputed by others [Wyllie et al 1972, Solez et al 1974, Molland 1978, Cuttino et al 1981, Wolf et al 1991b].

This proposed difference in mechanism for BEA-induced RPN suggests that it is not an ideal model for analgesic-induced RPN, particularly for studies aimed at preventing the development of this lesion by influencing the mechanisms involved. However, since the sequence of events leading to RPN with BEA mirrors that induced with analgesics and NSAIDS, BEA has been very important in establishing the site of initial damage and the early changes associated with this lesion, and is useful in evaluating techniques aimed at identifying RPN [Bach & Bridges 1982 & 1985, Mattingley et al 1984, Sabatini 1984, Wilks et al 1986, Bach et al 1986, Bach & Kwizera 1988, Gregg et al 1989a & b, 1990a & b].

The currently proposed mechanisms for analgesic and NSAID-induced RPN are metabolic activation and possibly inhibition of prostaglandin synthesis (Fig. 5.2) [Dunn & Zambraski 1980, Bach & Bridges 1984, Marnett et al 1984, Bach & Hardy 1985, Bach 1991, Kirkova et al 1992 & 1995, Hawksworth et al 1994]. Some investigators have also proposed an involvement for ischaemia, possibly due to the reported effects of these compounds on prostaglandins [Jackson & Lawrence 1978, Henrich et al 1978, Bach & Bridges 1984, Heyman et al 1988 & 1991].

Metabolic activation can occur via the prostaglandin synthetase (PGS) or lipoxygenase pathways. These pathways are involved in the co-oxygenation of xenobiotics in association with the metabolism of arachidonic acid (Figure 5.2). Analgesics and NSAIDS produce much of their therapeutic and adverse effects through the inhibition of prostaglandin synthesis, by inhibiting cyclo-oxygenase. Inhibition of cyclo-oxygenase may reduce vasodilation and lead to the metabolism of arachidonic acid (AA) via the lipoxygenase pathway (Figure 5.2), resulting in the production of both fatty acid peroxides which can act as substrates for the prostaglandin hydroperoxidase step of PGS, leading to subsequent lipid peroxidation. Metabolism of AA via

lipoyxygenase may also lead to the production of leukotrienes, which can have profound effects on renal haemodynamics.

Indomethacin and aspirin are both well-documented inhibitors of prostaglandin synthesis, and indomethacin has been shown to have an inhibitory effect on prostaglandin synthetase, lipoyxygenase and cyclo-oxygenase, present within the interstitial cells of the papilla [Molland 1978, Dunn & Zambraski 1980, Clive & Stoff 1984, Marnett et al 1984, Zenser & Davies 1984, Bach & Bridges 1985, Gregg et al 1989a, Hawksworth et al 1994]. The presence of high levels of polyunsaturated fatty acids in the interstitial cells predisposes these cells to lipid peroxidation in the presence of locally generated reactive intermediates. The reactive intermediates produced via PGS or lipoyxygenase therefore have the potential to cause lipid peroxidation and lead to cell death, as illustrated in Figure 5.2 [Bach & Bridges 1984]. Normally, reduced glutathione (GSH) protects the cell from alkylating agents (e.g. BEA) and lipid peroxidation but the medulla and papilla have low levels of endogenous GSH, so once reactive intermediates are generated these will not be readily inactivated (as shown in Figure 5.2) [Duggin 1980, Mohandas et al 1984, Gregg 1989a].

In comparison with analgesics and NSAIDS, ethoxyquin has also been shown to inhibit drug co-oxidation during prostaglandin synthesis [Kahl 1984]. Furthermore, the effects of ethoxyquin on prostaglandin production and cyclo-oxygenase and lipoyxygenase activity have been investigated, in renal medullary slices and homogenate respectively, and compared with the effects of NSAIDS, particularly indomethacin [Zenser & Davies 1978, Bach & Bridges 1984, Miyazawa et al 1985]. Ethoxyquin, in common with NSAIDS, was shown to inhibit prostaglandin synthesis (particularly PGE₂) and cyclo-oxygenase activity. Ethoxyquin was in fact found to be a more potent inhibitor of these than indomethacin, and also slightly inhibited

lipoxygenase activity. The similarity of ethoxyquin to NSAIDs and analgesics in this respect, coupled with the fact that the sequence of pathological events leading to RPN follows the same course, suggests that ethoxyquin induces RPN by the same mechanism as analgesics. Ethoxyquin therefore has great potential as a model for further studying the mechanisms leading to RPN, and extrapolating the results to the lesions seen in man. As mentioned in the introductory chapter of this thesis, there is still much discussion and research involved in detecting RPN at an early stage and elucidating the mechanistic basis of this lesion. The use of ethoxyquin as a model may help in this, and provide important information for the identification and subsequent management of RPN in man, and in the design of new drugs which do not induce such effects.

5.4: SUGGESTIONS FOR FURTHER WORK

5.4.1: Technique-based

- Apply the techniques developed in these experiments to general toxicity studies where appropriate. The perfusion techniques developed have already been successfully used in regulatory and investigative toxicity studies in these laboratories to examine effects in the brain (using whole body perfusion) and the kidney (using whole body and local perfusion, where only one kidney was perfused).
- Evaluate the application of a “urinary screen” (as recommended above) for future studies with possible renal toxins. If this proved valuable, urine samples could then be collected at intervals throughout a study to identify any renal effects early in the course of treatment, rather than collecting samples only at the end of a study as is currently the case.
- Evaluate the application of SEM, already used for the kidney, to investigations involving brain tissue (depending on the site of interest), e.g. SEM combined with latex corrosion casts may be useful for investigation of the vasculature in the brain, as used by Coyle [1976 & 1978].

- The combination of SEM and correlative histology proved very useful in these experiments and is applicable to any tissue; use this combination of techniques in future toxicity studies where changes on epithelial or endothelial surfaces are of interest (e.g. bile duct changes in the liver, glomerular effects in the kidney, endothelial changes).
- Investigate the use of TEM for examination of urinary sediments. This technique, used by Mandal and co-workers [1985 & 1988], may have helped in the identification of cell types present in the sediments that were not discernible by light microscopy. Similarly, SEM may also be of use in studying the appearance of these cells. However, such techniques (particularly TEM) would be more time-consuming than the standard light microscopy preparation and assessment, and may therefore not be feasible for routine use in toxicity studies.

5.4.2: Further development of ethoxyquin / Investigation of mechanisms

- Investigate ethoxyquin as a chronic model for RPN, to enable the development of chronic RPN and the long term effects of treatment to be studied. The results could then be related to the effects seen in man with analgesic nephropathy, which is primarily a chronic lesion.
- Use histochemical and immunocytochemical techniques, e.g. lipid staining methods in kidney sections, renal antibodies to specific cell types in kidney sections or possibly urine [as suggested by Bomhard et al 1994], to investigate the lesion induced with ethoxyquin and compare the results with the other models. TEM and SEM provided very detailed information about the early development of the lesion in these experiments, and the histochemical changes associated with RPN in other models are well-documented [Hill et al 1972, Gregg et al 1989c & 1990a, Bach et al 1983 & 1991], so the use of histochemical techniques may not have provided any additional information. However, the application of these techniques may be useful if further studies were conducted with ethoxyquin as a chronic model for RPN, since in this case they could provide

extra information from paraffin sections to supplement the standard H&E sections in the absence of ultrastructural investigations.

- Further investigate the effects of RPN induced with ethoxyquin on renal prostaglandins by conducting measurements of urinary prostaglandins, specifically PGE₂, following ethoxyquin treatment and comparing the results with the effects of indomethacin [Melendez & Reyes 1982, Clive & Stoff 1984, Rainsford et al 1988]. However, since it is unclear whether the known effects of analgesics on prostaglandins are primarily involved in the development of RPN, this information, although interesting, may not help in further investigating the mechanisms involved.
- Further investigate the effects of ethoxyquin on lipid peroxidation. Lipoyxygenase measurements may provide useful further information regarding the pathogenesis of this lesion and allow further comparison of this model with the effects induced by indomethacin and aspirin [Miyazawa et al 1985, Kirkova et al 1992 & 1995], and possible extrapolation to the effects of analgesics in man. Furthermore, investigation of the effects of lipoyxygenase and/or PGS inhibitors (hydroperoxidase) on the development of RPN induced with ethoxyquin and indomethacin would provide the opportunity to investigate whether one or both of these pathways is involved in the development of RPN (as suggested by Hawksworth et al [1994] and Stewart et al [1994]). Such investigations would also provide further information regarding the mechanisms involved in the development of RPN with ethoxyquin and consequently its suitability as a model for RPN.

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APPENDIX 1: STAINING METHODS AND BUFFER RECIPES

Haematoxylin and eosin stain for histological sections:

<u>Reagent</u>	<u>Time</u>
Xylene	2 x 5 minutes
100% alcohol	2 minutes
95% alcohol	1 minute
70% alcohol	1 minute
Distilled water	1 minute
Harris' haematoxylin	5 minutes
Distilled water	1 minute
1% acid alcohol	1 second
Distilled water	30 seconds
Scotts tap water (ammonia water)	2 minutes
Distilled water	30 seconds
Eosin	3 minutes
95% alcohol	2 x 30 seconds
100% alcohol	2 x 1 minute
Xylene:100 alcohol (50:50 mix)	2 minutes
Xylene	2 x 4 minutes

Mount in DPX mountant.

Papanicolau stain for urine sediments

<u>Reagent</u>	<u>Time</u>
95% alcohol	1 minute
70% alcohol	1 minute
Distilled water	1 minute
Harris' haematoxylin	3 minutes
Distilled water	1 minute
Acid alcohol	1 second
distilled water	30 seconds
Scotts tap water	2 minutes
Distilled water	30 seconds
OG6 stain	1 min 30 seconds
95% alcohol	30 seconds
EA50 stain	1 min 30 seconds
95% alcohol	2 x 30 seconds
100% alcohol	2 x 1 minute
Xylene: alcohol (50:50)	2 minutes
Xylene	2 x 4 minutes
Mount in DPX mountant	

0.2M Sorensens phosphate buffer (stock concentration)

Solution A: Disodium hydrogen orthophosphate (anhydrous); 28.4g in 1.0L distilled water.

Solution B: Sodium dihydrogen orthophosphate (anhydrous); 24g in 1.0L distilled water.

For 0.1M phosphate buffer: 70mL solution A + 30mL solution B, add 100mL distilled water, adjust pH to 7.4.

Toluidine Blue stain for semi-thin sections

<u>Reagent</u>	<u>Time</u>
1% toluidine blue in 1% sodium tetraborate	45 seconds.
Dry on hotplate at 60°C and coverslip with DPX mountant	

Methylene blue/Azure II stain for semi-thin sections

<u>Reagent</u>	<u>Time</u>
Methylene blue/Azure II	90 seconds at 60°C
water wash	15 seconds
Basic Fuchsin	45 seconds
Water wash	30 seconds
dry on hotplate at 60°C, coverslip with DPX mountant.	

Uranyl acetate/lead citrate stain for ultrathin sections

<u>Reagent</u>	<u>Time</u>
Saturated uranyl acetate in 50% alcohol	5 minutes
Distilled water	3 x 30 seconds
Dry on fibre-free tissue	30 seconds
Reynolds lead citrate solution	5 minutes
Distilled water	3 x 30 seconds
Dry on fibre-free tissue, store in grid box.	